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[GANN, 47, 1~14; March, 1956]

STUDIES ON THE CHROMOSOMES OF TUMOR AND NORMAL CELLS OF RATS. ADVOCATION OF THE "GENONEME" THEORY. (With Plates I-V)

ISAMU USUBUCHI and TETSUO KOSEKI

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INTRODUCTION

The theory that each species of living being has its constant chromosome complex both in number and in shape, is introduced in the first pages of the most textbooks of biology and heredity, but in other pages are noted variations of the chromosome number of the same species as the results of fragmentation, fusion, supernumerary chromosome, non-disjunction or polyploidy.

Variations of the chromosome number of malignant tumor cells have been reported by many pathologists. The Yoshida sarcoma,[1] an ascites sarcoma of rat, is an excellent material for the study of chromosomes. Makino[2] and others reported that the variation of the chromosome number of the Yoshida sarcoma takes place around 40, showing a gradual fluctuation both above and below that number, and further that the tumor cells with subdiploid chromosomes, 40 or thereabout in number, are significant in connection with the growth of the tumor. They reported that the existence of a prominent large V-shaped element is specially notable, because the chromosomes of the host animal[3] (**Rattus norvegicus**) are 42 in number and all are of rod-type.

Sato[4] also studied the chromosomes of the Yoshida sarcoma using tumor cells proliferated in the peritoneal cavity of the rat that had been transplanted with a single cell. He concluded from the experiment that the number of chromosomes varied widely between 35 and 50, the regular number of the spermatid of rats 42 being the center, and that V-shaped chromosomes were noticed in 42 out of 100 nuclear plates.

After the appearance of the studies on the chromosomes of the Yoshida sarcoma in considerable number, several ascites sarcomas, namely, the MTK sarcoma,[5] the Hirosaki sarcoma,[6,7] the Takeda sarcoma[8] and the Usubuchi sarcoma have been successively established. Makino and others reported that the chromosome number of the MTK sarcoma and the Hirosaki sarcoma varied around 40 with several prominent large V-shaped chromosomes. Yosida[9] reported that most of the Takeda sarcoma cells showed tetraploidal variation, and that there existed a

strain of tumor cells having a subtetraploid complex of chromosomes.

Usubuchi and others[10] reported that the chromosome number of the Hirosaki sarcoma varied around 40, and that a new tetraploidal strain of the Hirosaki sarcoma developed from the ordinary diploidal one. Koseki and Usubuchi[11,12] also found the chromosome number of the Usubuchi sarcoma varied with two peaks of about 40 and 75.

The authors studied further the chromosomes of somatic cells (lymphoblasts and macrophages) and male germ cells of rats in order to clarify the relation between the chromosmes of normal and tumor cells.

Materials and Methods

The Hirosaki sarcoma[6] was established by the intraperitoneal transplantation of a tumor that developed spontaneously in the cervical lymph nodes of a male rat of unpurified strain in 1951 in our laboratory. The characteristics of this tumor are similar to those of the Yoshida sarcoma and the MTK sarcoma. Cytological examination of these tumors showed that it was most suitable to classify them into lymphosarcoma showing diploidal chromosomes. During the successive intraperitoneal transplantations of this diploidal Hirosaki sarcoma a new type of tumor with tetraploidal chromosomes developed that was looked for as a transitional type to reticulosarcoma.

The Usubuchi sarcoma[11] was established by the intraperitoneal transplantation of a spindle cell sarcoma which developed at the site of methylcholanthrene injection in a male rat of unpurified strain in our laboratory (1952). The histological characteristics of this tumor changed gradually from spindle cell sarcoma to reticulosarcoma during the successive intraperitoneal transplantations.

The chromosmes of the Hirosaki sarcoma, the Usubuchi sarcoma and ascites macrophages were studied by the ordinary squash method[13] of staining with acetic dahlia violet. In order to study the chromosomes of lymphoblasts and male germ cells, lymph nodes or testis were cut off and crushed into pieces, which were stained by the same method as in the cases of ascites sarcoma cells.

Experimental Results

1) **Hirosaki sarcoma.** Usubuchi[10] and others found a tetraploid type of the Hirosaki sarcoma during the successive intraperitoneal transplantations of the ordinory diploid type of the Hirosaki sarcoma. From the former diploid type developed twice, that is, the reciprocal transformation between diploid type and tetraploid type was proved where the chromosome number of the diploid type of the Hirosaki sarcoma varied widely, showing a normal curve with the peak at about 37-38 and the chromosome number of the tetraploid type of the Hirosaki sarcoma

also varied widely, showing a normal curve with the peak at about 70–72. (Table 1, Fig. 1.)

Table 1. Distribution of chromosome numbers of the Hirosaki sarcoma.

Type	Case no.	Rat no.	Days after transplantation	Diploid cells			Tetraploid cells			Octaploid cells	
				No. of cells obs.	Distribution of chromosome numbers	Peak of curve	No. of cells obs.	Distribution of chromosome numbers	Peak of curve	No. of cells obs.	Distribution of chromosome numbers
Diploid type	1	SS 729	5	100	34～46	39					
	2	SS 704	8	98	31～47	38	2	61～ 72			
	3	SS 483	4	98	22～49	40	2	59～ 66			
	4	SW 498	4	98	25～52	38	2	67～ 76			
	5	SS 469	14	90	12～52	37	10	55～ 92			
	6	SS 469	23	84	22～49	36	14	62～ 79		2	121～145
	7	STM 119	3	97	30～43	37	3	62～ 76			
	8	STN 44	5	48	28～42	37	2	74～ 78			
Transitional type	1	STM 59	5	14	30～45		11	62～ 79			
	2	STM 78	3	61	34～48	38	39	54～ 81	72		
	3	STM 84	3	76	29～46	37	24	53～ 83	70		
Tetraploid type	1	ST 1	53	4	31～40		90	52～ 88	73	6	97～140
	2	STL 42	6	2	35～42		23	65～ 89	73		
	3	STL 51	4				25	58～ 82	74		
	4	STL 69	4	2	36		97	52～ 81	70	1	179
	5	STL 58	5				25	68～ 85	76		
	6	STL 76	9		34～39		97	54～ 92	74	1	136
	7	STL 92	8				99	53～100	70	1	152
	8	STL 120	2				100	53～ 91	70		
	9	STL 149	8				98	50～102	70	2	126～156
	10	STL 169	7				99	54～ 82	71	1	116
	11	STL 189	7				48	59～ 85	73	2	101～143
	12	STM 3	6				100	49～ 91	73		
	13	STM 10	9				96	52～ 86	71	4	108～134
	14	STM 40	8	2	41～45		23	61～102	75		

Morphological analysis of the chromosomes of the Hirosaki sarcoma in both types revealed that the chromosome complex consisted mostly of rod-shaped chromosomes. Sometimes large V-shaped or large J-shaped chromosomes were recognized, but they could not be accepted as indispensable elements because chromosome complex without large V-shaped or J-shaped chromosomes was often observed. (Figs. 5–17, Figs. 66-69.)

2) **Usubuchi sarcoma.** Koseki and Usubuchi[12] studied the chromosomes of the Usubuchi sarcoma. At the beginning of the successive intraperitoneal transplantations the chromosome number of the Usubuchi sarcoma distributed widely showing two normal curves of diploid and tetraploid types, of which the former was higher than the latter. During the successive transplantations the tumor cells of tetraploid type gradually increased in number, and so now most of the tumor cells showing chromosomes of tetraploid type. The chromosomes of the diploid type of the Usubachi sarcoma distributed widely showing a normal curve with the peak at about 40, and those of the tetraploid type with the peak at about 75. (Table 2, Fig. 2.)

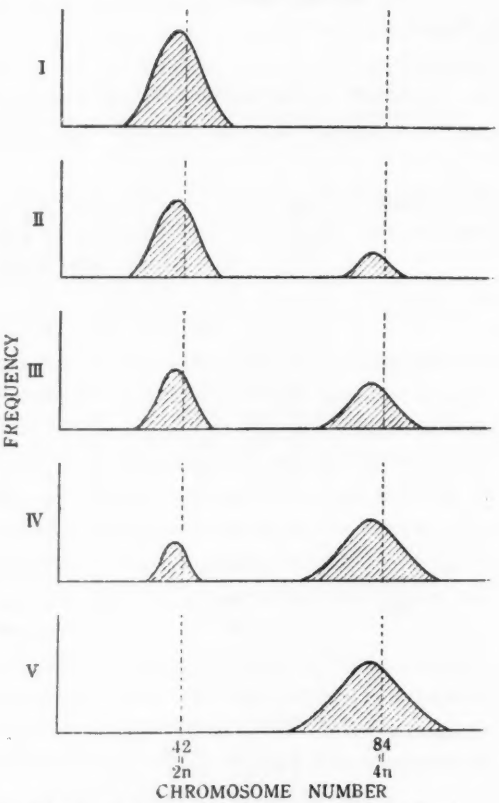

Fig. 1. Schematic exhibition of the distribution of chromosome numbers in various types of the Hirosaki sarcoma.

On morphological analysis of the chromosomes of the Usubuchi sarcoma in both types it was found that the chromosome complex consisted mostly of rod-shaped chromosomes. Large J-shaped or large V-shaped chromosomes were recognized in a certain percent of mitotic tumor cells. (Figs. 18-29, Figs. 70-72.)

3) **Lymphoblasts.** Koseki[14] studied the chromosomes of the lymphoblasts which are the main component of mitotic cells of the germ center of lymph nodes of rats. He found that the chromosome number of the lymphoblasts varied around 40. It was difficult to determine the exact number of the chromosomes because of the uncertain contour of one or two chromosomes of the chromosome complex. But in 10 mitotic cells the chromosome number could be exactly counted. The chromosome number varied between 25-45. (Table 3.)

The chromosome complex consisted mostly of rod-shaped chromosomes, but one large V-shaped chromosome was seen in each of 2 mitotic cells and one or two

Table 2. Distribution of chromosome numbers of the Usubuchi sarcoma.

Type	Case no.	Rat no.	Days after transplantation	Diploid cells			Tetraploid cells			Above tetraploid cells	
				No. of cells obs.	Distribution of chromosome numbers	Peak of curve	No. of cells obs.	Distribution of chromosome numbers	Peak of curve	No. of cells obs.	Distribution of chromosome numbers
Transitional type	1	P 75	13	43	25～57	40	52	60～ 92	75	5	97～350
	2	P 129	18	47	34～49	40	43	59～100	78	10	108～318
	3	P 164	11	60	34～48	41	36	66～ 88	80	4	130～146
	4	P 174	7	57	34～47	40	41	65～ 95	79	2	153～157
	5	P 177	3	40	32～50	41	54	64～ 92	80	6	115～165
	6	P 177	6	45	35～57	41	46	64～ 94	79	9	109～154
	7	P 181	6	39	38～52	41	57	67～ 92	81	5	113～163
	8	P 184	3	61	26～57	41	33	73～ 91	81	6	126～162
	9	P 184	6	39	37～60	43	60	70～ 97	80	1	114
	10	P 184	9	54	23～57	42	46	68～ 95	80		
	11	P 184	12	66	37～63	42	33	71～ 91	80	1	195
	12	P 184	15	36	34～55	44	61	70～ 93	83	3	108～155
	13	P 184	17	63	36～56	41	36	71～ 90	80	1	117
	14	P 205	9	7	40～51		88	64～ 91	79	5	101～162
	15	P 205	17	21	32～60		22	62～ 92		7	116～472
	16	P 214	8	8	29～46		85	63～ 88	77	7	115～172
	17	P 221	2	21	14～60		75	65～ 99	75	4	111～150
	18	P 221	6	6	41～63		89	66～ 91	76	5	112～165
Tetraploid type	1	P 255	6				47	67～ 94	75	3	113～168
	2	P 301	12				96	63～ 82	75	4	107～155
	3	P 313	9				98	61～ 81	73	2	142～149
	4	P 362	13	1	47		94	64～ 84	75	5	121～139
	5	P 366	6	1	45		94	65～ 92	74	3	107～134
	6	P 606	7	2	28～52		82	59～ 83	73	16	109～153

small or middle-sized V-shaped chromosomes in each of 4 mitotic cells. (Figs. 30–35.)

4) **Macrophages.** Usubuchi and Koseki[15] studied the chromosomes of macrophages which are the main component of ascites cells of rats. It was indeed difficult to decide the exact number of the chromosomes, but in 24 mitotic cells an exact calculation of the chromosome number was possible. The chromosome number varied between 40–50 in 22 mitotic cells and between 70–80 in 2 mitotic cells. (Table 4.)

The chromosome complex consisted mostly of rod-shaped chromosomes. Small or middle-sized V-shaped chromosomes, and middle-sized or large J-shaped

chromosomes were often observed. (Figs. 36–41.)

5) Male germ cells. Usubuchi and Kosek[16,17] reported twice on the chromosomes of male germ cells of normal rats. In the first examination the chromosome number was exactly ascertained in 29 mitotic cells among numerical mitotic cells with chromosome number around 20 or 40. The chromosome number varied between 9–84. (Table 5.)

In the second examination the chromosome number was exactly counted in 100 mitotic cells of diploid type which might correspond to spermatogonias. The chromosome number varied between 33–47 showing a normal curve with the peak at 42. (Table 6.)

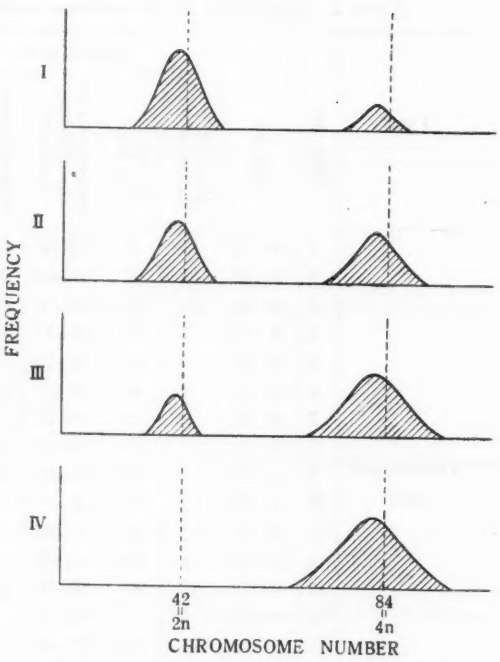

Fig. 2. Schematic exhibition of the distribution of chromosome numbers in various types of the Usubuchi sarcoma.

The chromosome complex consisted mostly of rod-shaped chromosomes both in diploid and haploid cells, although in haploid cells small rod-shaped chromosomes were more often seen than in

Table 3. Distribution of chromosome numbers of lymphoblasts of rats.

Chromosome number	25	31	37	38	42	43	44	45	Total
No. of cells obs.	1	1	1	1	2	2	1	1	10

Table 4. Distribution of chromosome numbers of macrophages of rats.

Chromosome number	42	43	44	46	47	48	49	50	53	73	76	Total
No. of cells obs.	4	4	3	3	1	2	2	1	2	1	1	24

Table 5. Distribution of chromosome numbers of male germ cells of rats (I).

Chromosome number	9	12	13	14	15	16	17	18	20	21	22	23	29	33	38	39	42	43	48	81	84	Total
No. of cells obs.	1	1	1	1	2	1	1	1	2	3	2	1	1	1	2	1	3	1	1	1	1	29

Table 6. Distribution of chromosome numbers of male germ cells of rats (II).

Chromosome number	33	36	37	38	39	40	41	42	43	44	45	46	47	Total
No. of cells obs.	2	1	2	6	9	10	9	26	16	8	8	2	1	100

diploid ones. Large or small V-shaped or J-shaped chromosomes were also seen. (Figs. 42–65, Figs. 73–78.)

Discussion

From the publication of the studies of many investigators on the chromosomes of the Yoshida sarcoma, the MTK sarcoma, the Hirosaki sarcoma and the Takeda sarcoma, it would seem to have been clarified that the chromosome number of tumor cells can not be confined to a constant value.

Makino and his co-workers[2,5,7,9] are of the opinion that there exists a peculiar strain cell in every kind of tumor cells, but the distribution of the chromosome number of every kind of tumor cells showing a normal curve, seems to be against their opinion. The data that the chromosome number of the Yoshida sarcoma, the MTK sarcoma and the Hirosaki sarcoma distributed widely showing a normal curve with the peak at about 40, can not be explained fully by the theory that the tumor cells with 42 chromosomes are ordinary and the others are abnormal. Moreover the data giveh by Sato[4] that the chromosome number of the Yoshida sarcoma proliferating from a single cell in the peritoneal cavity of a rat showed a normal distribution curve with the peak at about 42, can hardly be accepted by the strain cell theory.

The chromosome number of the ordinary Hirosaki sarcoma varied between 30–50 showing a normal curve with the peak at about 37–38. In one case of prolonged course of the ordinary Hirosaki sarcoma the mitosis decreased in number and tumor cells of large type which possessed tetraploidal chromosomes increased. Finally there appeared a new strain of the Hirosaki sarcoma which showed a variation of chromosome number between 60–80 showing a normal curve with the peak at about 70–72. From this tetraploidal Hirosaki sarcoma diploidal Hirosaki sarcoma developed twice. The data show the reciprocal relation between the diploidal and tetraploidal Hirosaki sarcomas. A part of the tetraploidal Hirosaki sarcoma cells showed an obvious phagocytizing action upon carbon particles, that is, a tetraploidal reticulosarcomatous tumor developed from a diploidal lymphosarcomatous tumor. These data may be explained by the results of our experiments on normal cells that lymphocytes, monocytes and histiocytes belong to the same strain of cells in postnatal life (Usubuchi).[18] (Fig. 3.)

At the beginning of the successive transplantations the chromosome number of the Usubuchi sarcoma distributed widely showing diploidal and tetraploidal normal

curves. At first the curve of the diploidal cells was higher than that of the tetraploidal ones, then gradually the tetraploidal cells increased in number and

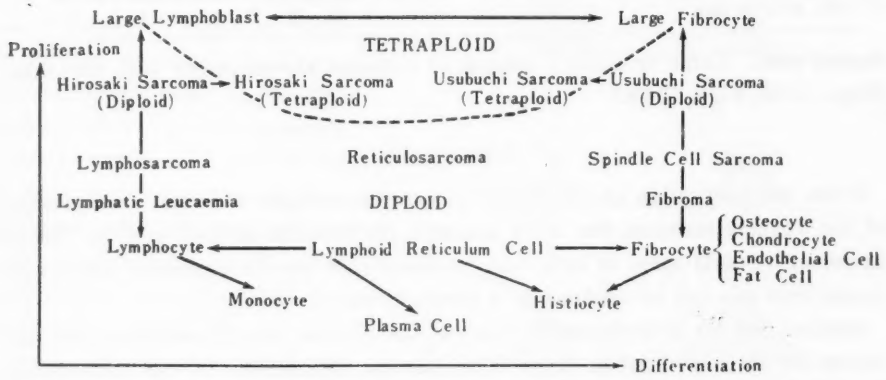

Fig. 3. Relation among the mesenchymal cells in postnatal life (Usubuchi). The dotted line differentiating diploid and tetraploid forms is drawn on the distribution of chromosome numbers of the Hirosaki sarcoma and the Usubuchi sarcoma.

now almost all the tumor cells are tetraploidal. The histological findings of the Usubuchi sarcoma changed gradually, at first it seemed most reasonable to classify the tumor into spindle cell sarcoma, but now, after having passed through the intermediate type it is most suitable to classify the tumor into reticulosarcoma. These data above mentioned may be explained by the results of our experiments on normal cells that fibrocytes and histiocytes belong to the same strain of cells in postnatal life (Usubuchi).[18] (Fig. 3)

From the above data concerning the chromosomes of the experimental ascites sarcomas of rats, it may be said to have been proved that the chromosome number is not constant. Is there any peculiar rule of mitosis in tumor cells which can not be found in normal cells? When a mitotic tumor cell is observed under a microscope, two daughter cells appear both of which possess the chromosome complex of the same number as the mother cell by the split formation in each chromosome of the mother cell. Morgan's theory can not permit the coexistence both of this fact and the data obtained by Sato that a single tumor cell proliferated in a short period to numerous tumor cells which showed every possible variation of chromosome number.

The chromosome number of normal cells has been reported to be constant generally and the variation of the number in some species was understood to indicate special meaning in its occurence. According to Tanaka[19] in the Makino laboratory, the tissue cells of various organs of young rats showed numerical variation of chromosome numbers from 36 to 84.

The chromosome number of lymphoblasts of the rat which are considered to be mother cells of lymphosarcoma as in the Yoshida sarcoma, the MTK sarcoma and the ordinary Hirosaki sarcoma, varied from 25 to 45. The chromosome number of macrophages of the rat which are thought to be mother cells of reticulosarcoma such as the present type of the Takeda sarcoma and the Usubuchi sarcoma, varied also widely from 42 to 76 showing two groups of diploidal and tetraploidal chromosomes.

According to Makino the chromosome number of the male germ cells of rats is constantly 42 in diploid and 21 in haploid cells. The results of our experiments on the chromosome number of the male germ cells of rats showed a considerable variation from 9 to 84, although most of the chromosomes appeared either around 40 or 20. Recently Kano[20] in the Makino laboratory recognized a variation of chromosome number of male germ cells under abnormal conditions.

As the original animals of the experimental sarcomas such as the Yoshida sarcoma, the MTK sarcoma, the Hirosaki sarcoma, the Takeda sarcoma and the Usubuchi sarcoma were rats, the studies on the chromosomes of normal cells have been performed in the same species, and the variation of the chromosome number of normal cells of rats have been confirmed. The experimental data shown above in rats can be expected in the same way in every species of living being when examined in detail as in rats. Morgan's theory that each species of living being has its constant chromosome complex both in number and in shape, must, therefore, be denied.

Theory of Genonemes

The authors here propose a new theory on chromosomes, namely that the chromosome number may vary in each species of living being. The opinion published heretofore that the homological genetic factors (genes) of fatherhood and motherhood are separated into their respective chromosomes, is according to the authors not valid, but they must rather conjugate together in one chromosome. If the chromosome complex be considered to be a long string as a whole in resting nucleus, and if it can be cut off freely in number and in shape in mitotic nucleus, the number and shape of chromosomes can vary freely without bringing about any change of genetic factors. On this occasion, in spite of the possibility of change of the number and shape of each chromosome, the characteristics of the chromosome complex as a whole is supposedly kept in each species of living being and in one kind of cell.

In the authors' theory that the chromosome is a long string consisting of homological genetic factors of fatherhood and motherhood in resting nucleus, every component of the chromosome which requires a combination of indispensable

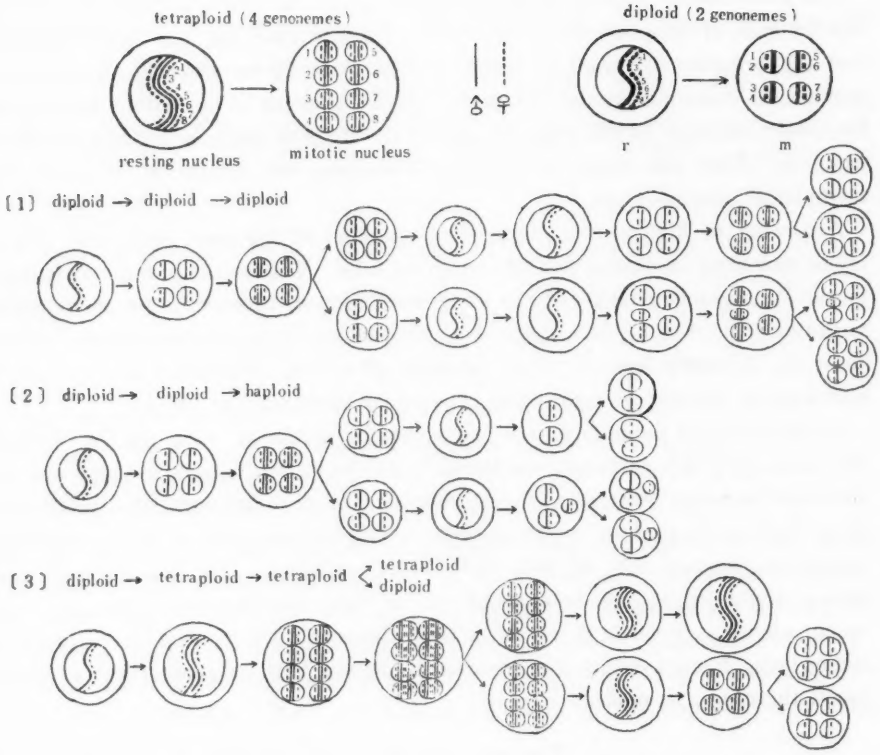

Fig. 4. Relation between chromosome number and polyploidy based upon our genoneme-theory.

genetic factors, is called "genoneme". (Fig. 4.)

Each chromosome of diploid cells consists of 2 genonemes corresponding to fatherhood and motherhood. In mitosis each genoneme multiplies twofold and there appear 4 genonemes which separate into two daughter cells. As the quantity of the chromatin of diploid cells suitable for mitosis is almost constant in the same species of living beings, the chromosome number can vary showing a normal curve with almost constant peak.

Each chromosome of tetraploid cells consists of 4 genonemes. In mitosis each genoneme multiplies twofold and there appear 8 genonemes, which separate into two daughter cells. The quantity of the chromatin of tetraploid cells adequate to mitosis is almost constant in the same species of living beings, so that the chromosome number can show variation with almost constant peak which has about twice as many as the number of the chromosomes of diploid cells.

If a daughter diploid cell produces mitosis without growing, genonemes separate into two haploid cells without multiplication. This type of mitosis is usually seen only in germ cells. The quantity of chromatin of haploid cells suitable for mitosis being almost constant in the same species of living beings, the chromosome number varies with almost constant peak which has about half the number of the chromosomes of diploid cells.

The formation of tetraploid cells from diploid cells can be explained through the mechanism that each genoneme of diploid cells multiplies twofold in resting nucleus.

When a daughter tetraploid cell produces mitosis without growing, genonemes separate into two diploid cells without multiplication.

Summary

1) The chromosome number of the tumor cells of rats was ascertained to be variable. Moreover, in the Hirosaki sarcoma a new tetraploid type developed from an ordinary diploid type, and also in the Usubuchi sarcoma a new tetraploid type from an original mixed type of diploid and tetraploid.

2) Again, the chromosome number of the normal cells of rats, for example, that of lymphoblasts, macrophages and male germ cells was proved to be inconstant.

3) A new theory of genonemes that explains the variation of chromosome number in the same species of living beings is here advocated.

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Explanation of Plates I—V

Plate I

Metaphase plates of Hirosaki sarcoma cells. Camela lucida, ×1500.

Fig. 5.——38 chroms, 1 middle-sized V, 1 large J and 1 middle sized J.
Fig. 6.——37 chroms, 1 large J.
Fig. 7.——41 chroms, 1 middle-sizes V.
Fig. 8.——40 chroms., 1 middle-sized V and 2 middle-sized J.
Fig. 9.——38 chroms., 1 large V, 1 middle-sized V and 1 middle-sized J.
Fig. 10.——38 chroms.
Fig. 11.——38 chroms., 1 large V.
Fig. 12.——77 chroms., 1 large J and 2 middle-sized J.
Fig. 13.——70 chroms.
Fig. 14.——76 chroms., 1 middle-sized V and 2 middle-sized J.
Fig. 15.——73 chroms., 1 large V.
Fig. 16.——84 chroms.
Fig. 17.——75 chroms., 1 large J and 1 middle-sized V.

Plate II

Metaphase plates of Usubuchi sarcoma cells. Camera lucida. ×1500.

Fig. 18.——38 chroms.
Fig. 19.——45 chroms.
Fig. 20.——46 chroms.
Fig. 21.——43 chroms., 1 large J.
Fig. 22.——42 chroms.
Fig. 23.——43 chroms.
Fig. 24.——81 chroms., 1 middle-sized J.
Fig. 25.——86 chroms., 1 large J and 2 middle-sized J.
Fig. 26.——89 chroms.
Fig. 27.——78 chroms.
Fig. 28.——76 chroms., 1 large V.
Fig. 29.——80 chroms.

Plate III

Metaphase plates of lymphoblasts of rats. Camera lucida. ×1500.

Fig. 30.——25 chroms.
Fig. 31.——38 chroms.
Fig. 32.——42 chroms.
Fig. 33.——45 chroms.
Fig. 34.——42 chroms., 2 small V.
Fig. 35.——43 chroms., 2 middle-sized V.

Metaphase plates of macrophages of rats. Camera lucida. ×1500.

Fig. 36.——42 chroms.
Fig. 37.——46 chroms.
Fig. 38.——48 chroms., 1 middle-sized J.
Fig. 39.——43 chroms.
Fig. 40.——44 chroms., 1 middle-sized V.
Fig. 41.——76 chroms.

Plate IV

Metaphase plates of male germ cells of rats. Camera lucida. ×1500.

Fig. 42.——33 chroms.
Fig. 43.——36 chroms.
Fig. 44.——37 chroms.
Fig. 45.——38 chroms.
Fig. 46.——39 chroms.
Fig. 47.——40 chroms., 1 middle-sized V.
Fig. 48.——41 chroms.
Fig. 49.——42 chroms.
Fig. 50.——43 chroms.
Fig. 51.——44 chroms.
Fig. 52.——45 chroms.
Fig. 53.——46 chroms.
Fig. 54.——47 chroms.
Fig. 55.——84 chroms.
Fig. 56.——39 chroms., 1 large J and 1 small V
Fig. 57.——43 chroms., 1 middle-sized V.
Fig. 58.—— 9 chroms.
Fig. 59.——17 chroms., 1 middle-sized V.
Fig. 60.——21 chroms.
Fig. 61.——21 chroms.
Fig. 62.——22 chroms.
Fig. 63.——22 chroms.
Fig. 64.——23 chroms.
Fig. 65.——23 chroms.

Plate V

Photomicrographs showing metaphase plates of tumor and male germ cells of rats. ×1000.

Fig. 66.——Hirosaki sarcoma cell, 41 chroms.
Fig. 67.—— 〃 , 40 chroms.
Fig. 68.—— 〃 , 73 chroms.
Fig. 69.—— 〃 , 76 chroms.
Fig. 70.——Usubuchi sarcoma cell, 70 chroms.
Fig. 71.—— 〃 , 70 chroms.
Fig. 72.—— 〃 , 79 chroms.
Fig. 73.——Male germ cell of rat, 33 chroms.
Fig. 74.—— 〃 , 40 chroms.
Fig. 75.—— 〃 , 42 chroms.
Fig. 76.—— 〃 , 42 chroms.
Fig. 77.—— 〃 , 46 chroms.
Fig. 78.—— 〃 , above 78 chroms.

要　旨

白鼠の腫瘍及び正常細胞の染色体に関する研究・遺伝糸説の提唱

臼淵　勇・小関哲夫
(弘前大学医学部病理学教室)

1)　白鼠の腹水肉腫である弘前肉腫及び臼淵肉腫の染色体には吉田肉腫と同様に，一定の数と形を見出すことはできなかった。弘前肉腫においては一般の diploid 型から累代中に tetraploid 型が主体の新しい系統を生じた。また臼淵肉腫では当初は diploid 型と tetraploid 型が略均等にみられたが，累代につれて tetraploid 型が増加し，ほとんどが tetraploid 型と変った。

2)　白鼠の体細胞である淋巴芽球，大食細胞及び睾丸性細胞の何れの染色体においても一定の数と形を見出すことはできなかった。淋巴芽球は diploid 型であり，大食細胞は主として diploid 型で，一部は tetraploid 型であり，睾丸性細胞は haploid 型及び diploid 型が主体で，一部に tetraploid 型をも認めた。

3)　父性または母性の不可欠の遺伝因子の組合せを遺伝糸 genoneme とし，染色体は相同の父性及び母性の遺伝糸が結合して成立するもので全体として 1 本の長い糸状のものであって，これが自由に切断されて染色体の数と形が変化しても遺伝因子に変化はおきないという仮説を提唱した。


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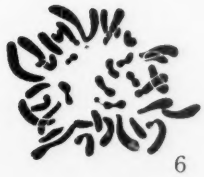
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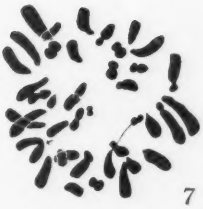
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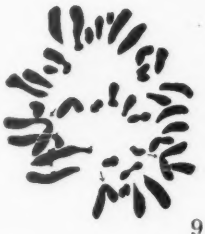
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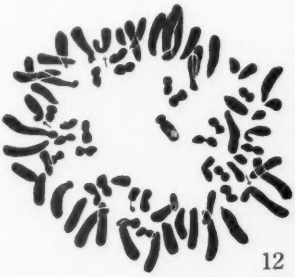
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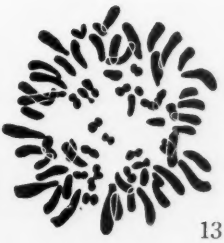
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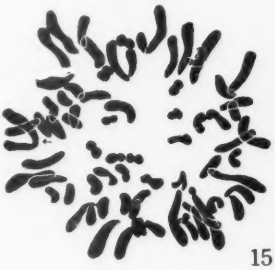
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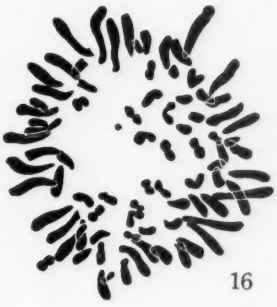
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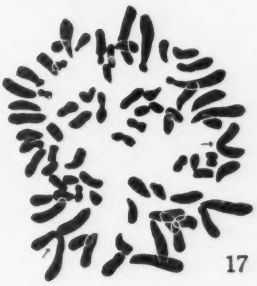
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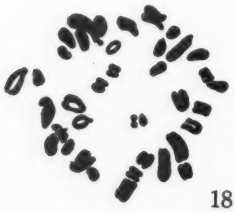
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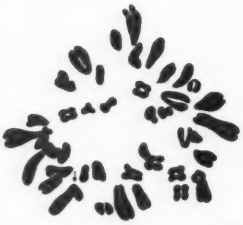
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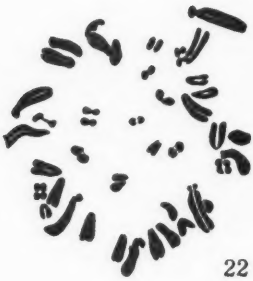
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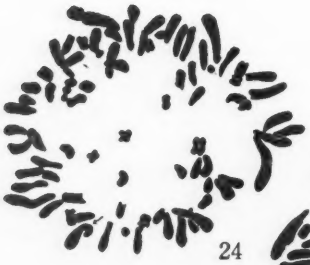
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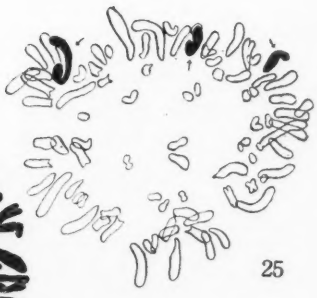
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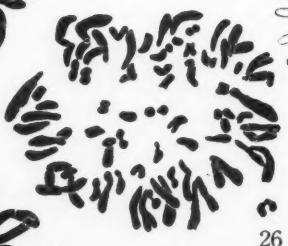
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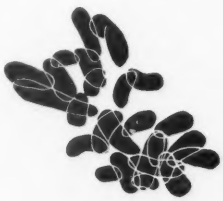

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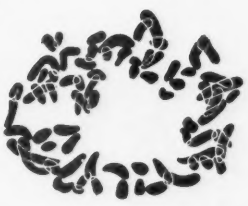

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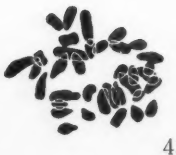
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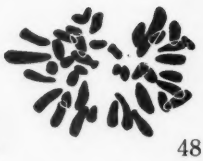
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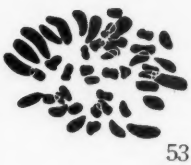
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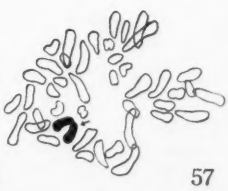
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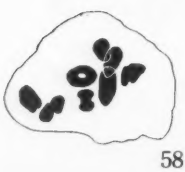
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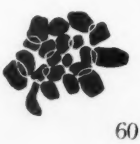
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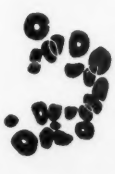
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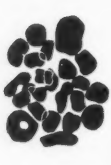
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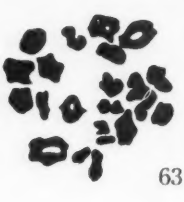
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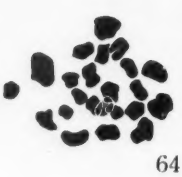
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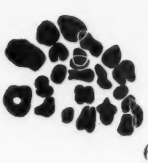
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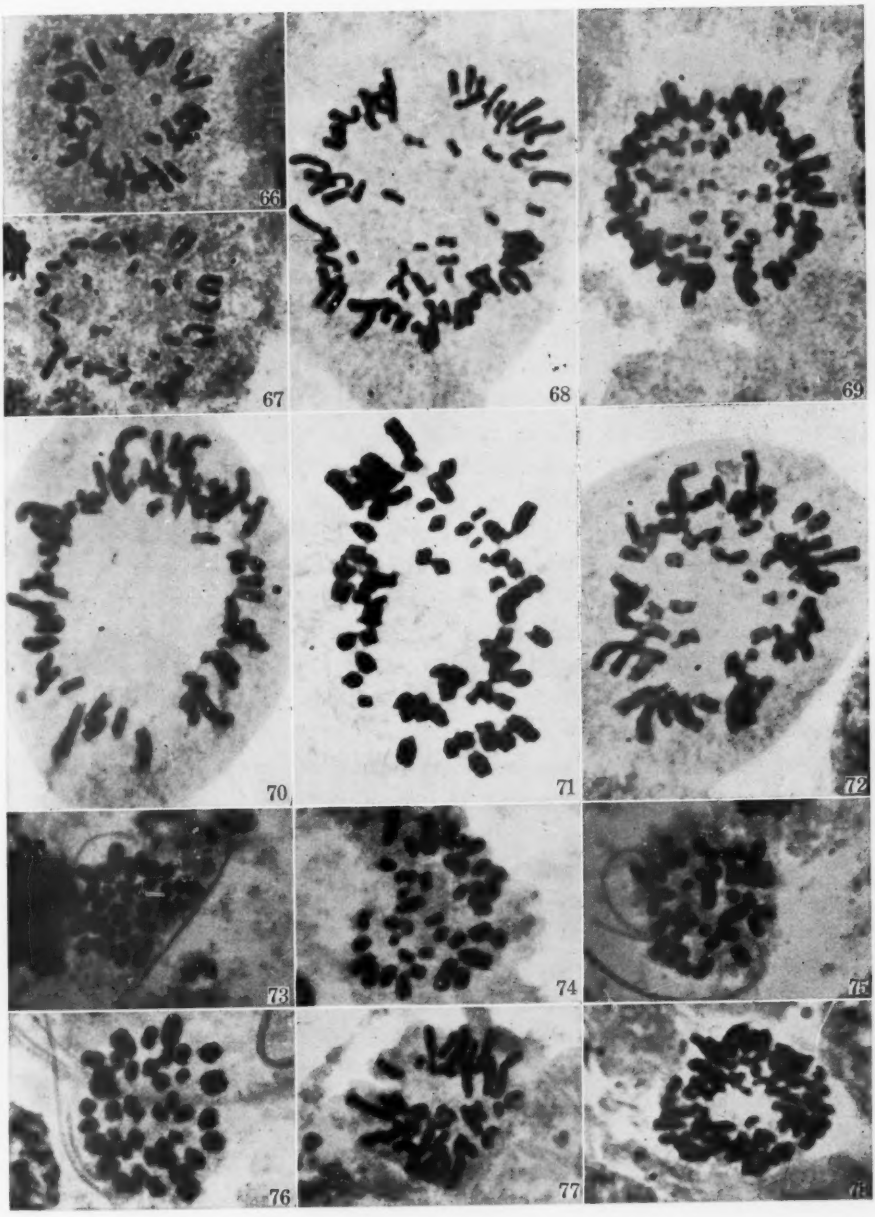
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[GANN, 47, 15~22; March, 1956]

ON THE WATANABE ASCITES HEPATOMA, A NEW ASCITES TUMOR OF RATS, PRODUCED AFTER THE APPLICATION OF HOT WATER

(With Plates VI and VII)

FUMITOMO WATANABE and AKIRA TONOMURA

(Animal and Medical Institute in Ohmura, Nagasaki University School of Medicine, and Zoological Institute, Faculty of Science, Hokkaido University)

It is in recent years that much progress has been made in studies on the chromosome cytology of tumors, especially with the use of ascites tumors of rats or mice, thanks to extensive studies of Makino and his collaborators (Makino 1952 a, b, Makino & Kanò 1951, 1953, 1955, Makino & Nakahara 1953 a, b, Makino & Tanaka 1953 a, b, Makino & Tonomura 1955, Tanaka & Kanò 1951, Tonomura 1954, Umetani 1953, Yosida 1954 a, b, etc.), Levan & Hauschka (1952, 1953), Hauschka (1953), Bayreuther (1952), Kaziwara (1954) and Sachs & Gallily (1955). It was established that there is a population (or populations) of tumor cells which contribute to the growth of the tumor. Those tumor cells are characterized by a particular karyogram constituted of characteristic chromosomes which persist in their individuality by dividing in regular manner. Thus the concept of the stemline-cells from which the tumor propagates was developed on the basis of the morphological and statistical analyses of the chromosomes (Makino 1952, Makino & Kano 1955, Makino & Tonomura 1955, Levan & Hauschka 1952, 1953, Hauschka 1953).

In the course of studying the cytological changes in the somatic cells of rats through repeated subcutaneous injections with hot water at 72°C, Watanabe, one of the present authors, met with the formation of a new transplantable tumor in the liver of a white rat. Transformation of this solid tumor into an ascites form was attempted resulting in the establishment of a new transplantable ascites tumor (Watanabe & Matunaga 1954, Watanabe & Azuma 1955).

The present paper describes the procedure by which this tumor was produced, together with the general features of the new tumor. In addition some preliminary cytological aspects of this ascites tumor will be given for comparison with those of several other ascites tumors of rats.

The authors wish to express their sincere gratitude to Professor Sajiro Makino for his direction and for improvement of the manuscript for publication. To Mrs.

K. Tanaka and Mr. H. Nakahara the authors are also greatly indebted for valuable advices and kind aid. Financial aid from the Scientific Experimental Research Fund of the Ministry of Education is gratefully acknowledged here.

I. Production of the Tumor

Procedure through which the present tumor arose is described as follows: ten white rats of mixed breed received subcutaneous injections 2 times a week for a year with hot water at 72°C. About 3 months after beginning the injections, a solid tumor of walnut-size developed in the liver of one of the rats (Fig. 1). At autopsy, some nodular metastases were observed on the infra-diaphragmatic surface, but at that time, there was present no ascites in the peritoneal cavity. Some tissue pieces derived from the original tumor were grafted subcutaneously in some rats. This induced the development of a large massive tumor in each rat. On the other hand, intraperitoneal inoculation of saline suspension of the crushed tumor tissue pieces was attempted in some rats. This resulted in the production of freely suspended tumor cells in the ascites of the peritoneal cavity. Successive transfers of this ascites tumor have been made with success from rat to rat.

The senior author examined the histological features of the original tumor, also the massive solid tumors produced after the second transplantation. The original tumor tissue was found to contain at least three kinds of cells forming the tissue. One of them is provided with the tumor cells which morphologically simulate the hepatic cells surrounding them (Figs. 2-3). The cytoplasm of these cells is generally vesicular and basophilic. The nuclei are round or oval in shape. Nucleoli, generally one or two in number, are present in the nucleus. The second type of cells is of small size simulating Kupffer cells (Fig. 2). They are found intermingled with the tumor cells of the former type. They are highly basophilic, being characterized by nuclei of oval or sometimes spindle shape. The third type comprises syncytial polynuclear giant cells having 3 to 15 nuclei; they occur among the tumor cells of other types (Fig. 3), being few in number. The stroma are hardly recognized. Mitotic figures are frequent in occurrence. The tumor tissue is sharply distinguishable from the non-neoplastic hepatic tissue, being separated by a distinct wall of the cysticercus cyst of the liver, as shown in Figure 4. The hepatic cells lining the inner layer of the cysticercus wall highly resemble neoplastic cells in general feature undergoing a nodular proliferation.

The nuclei and cytoplasm of tumor cells after subcutaneous transplantation tend to increase in volume as compared with those of the original tumor. Polymorphism of the nucleus begins to increase at about the tenth transplant generation and has continued onward. Metastases are usual in the mediastinal lymph nodes of the host.

The massive tumor tissue is usually divided by fine capillaries into the so-called tumor cell-nests specific to the hepatoma (Fig. 5). This histological feature impressed students that the present tumor is a type of the atypical hepatoma. The authors propose to designate this tumor by the name "Watanabe ascites hepatoma" in the following.

II. General Characteristics of the Watanabe Ascites Hepatoma

The general characteristics of the Watanabe ascites hepatoma were studied by the junior author; the descriptions will be made in comparison with those of the Yoshida sarcoma and MTK-sarcomas.

1. **Tumor cells:** The tumor cells are classified as regard to their size into the following three types; small-sized cells, medium-sized cells and large-sized cells. The diameters of cells are 20–21 μ, 23–28 μ and more than 30 μ respectively for the small-sized, medium-sized and large-sized tumor cells. Generally speaking, the nuclei are oval or kidney shaped, and lie in one side of the cell. The number of nucleoli varies from one to five. In the wide space of the cytoplasm, distinct granules are visible when observed in living state; they show a semi-circular arrangement converging to a center. They are slightly stained with Giemsa's stain, but the application of supravital technique with neutral red or toluidine blue render visible some of them.

2. **Transplantability:** The Watanabe ascites hepatoma is highly sensitive to Gifu rats, showing the transplantability at more than 90 percent. But, rats of other stocks are less or nonsusceptible to this tumor. Detailed data are shown in Table 1.

Table 1. Comparison of transplantability of the Watanabe ascites hepatoma in five different strains of rats.

Rat stocks	Number of animals transplanted	Number of tumor animals died	% of transplantability
Gifu (g)	32	30	93.8
Gifu-black (g-a)	10	3	30.0
Gifu Wistar	5	1	20.0
Wistar (w/Ma)	7	0	0
Wistar-King (WKA)	5	0	0

3. **Life span of the tumor-bearing animals:** The whole life span ranging from the day of transplantation of the tumor to the death of the host animal was found to be longer in the Watanabe ascites hepatoma than in the Yoshida sarcoma or MTK-sarcomas (Makino 1952, Tanaka & Kanò 1951, Umetani 1953, Tonomura

1954). The death of the diseased animal occurs in 12 days or more on the average, the extreme case being 23 days.

4. Rate of mitosis in a transplant generation: The mitotic rate was observed as similar to that in the Yoshida sarcoma and MTK-sarcomas. The data are given in Table 2. Generally, the mitotic rate strikingly increases during the early

Table 2. Daily frequency of mitotic cells in a tumor rat.
The percentage of dividing cells was calculated on the basis of 2000 cells per day in the observation through a transplant generation.

Days after transplantation	1	2	3	4	5	6	7	8	9	10	11	12
% of dividing cells	2.8	3.8	3.3	2.8	2.0	2.1	1.5	1.4	0.4	0.5	—	—

part of a transplant generation and decreases with time towards the latter part of the life span. On the 8th or 9th day after transplantation, the tumor cells undergo damage in large numbers, resulting in a sudden decrease in number of the tumor cells. At that time the dividing cells are scarcely present in the peritoneal fluid. In this condition the diseased animal dies.

5. Chromosome number: Fluctuation of the chromosome number occurs within a very wide range from about 20 to approximately 300 in the Watanabe ascites hepatoma. This ascites hepatoma is a type of tumor of mixed stem-cell lines, since it is represented by at least three different neoplastic populations provided with subtetraploid, subdiploid, and subhexaploid stem-cells, respectively.[1] Among these populations, the subtetraploid population of tumor cells is most predominant in occurrence, appearing at approximately 50 percent. The subdiploid population ranks next, followed by the subhexaploid population in low frequency. The modal variations of chromosome numbers are 80 to 90 for subtetraploid cells, 40 to 44 for subdiploid cells, and 116 to 125 for subhexaploid cells. The chromosome complexes are specific to these cell types; the numbers of the constituting elements, rod-, V- and J-shaped ones, are characteristic to each type. Examples are shown in Figures 6 to 11. The results of detailed morphological analysis of the chromosomes which is now in progress, will be published elsewhere in the near future.

6. The behaviour of the tumor cells in a transplant generation: Through a transplant generation of the Watanabe ascites hepatoma, the daily frequencies of the subdiploid, subtetraploid, subhexaploid cells together with other aneuploid cells were observed on the basis of the study of 100–150 cells per day. On the 1st day after transplantation, generally, the large-sized tumor cells make their

1) In addition to them, there occur tumor cells showing aneuploid chromosome numbers in low frequency. It seems probable that they are not cells forming a stem-line.

predominant appearance in the ascites, together with a certain number of giant cells. As shown in Table 3, the subtetraploid cells appears at the highest rate,

Table 3. Daily frequencies of subdiploid, subtetraploid, subhexaploid and other aneuploid cells at metaphase as observed in a transplant generation of Watanabe ascites hepatoma.

Days after trans-plantation	2n-type cells (%)	4n-type cells (%)	6n-type cells (%)	other cells (%)	Total No. of metaphase cells obs.
1	13.6	42.7	20.9	22.8	110
2	42.3	34.6	7.7	15.4	113
3	34.0	44.0	12.0	8.0	135
4	43.6	33.6	15.5	7.3	110
5	46.7	36.7	10.8	5.8	120
6	34.5	43.1	17.2	5.2	107
7	10.3	68.4	10.6	10.7	101
8	7.7	64.3	12.7	15.3	58
9	—	—	—	—	—
10	—	—	—	—	—

and the subhexaploid cells rank next. On the 2nd or 3rd day after transplantation, the small cells comprising subdiploid chromosomes show a remarkable increase with the decrease of the subhexaploid cells. During this period, the subtetraploid cells were found to be nearly similar. On the 7th or 8th day after transplantation, the mitotic figures of subdiploid cells suddenly decrease. The majority of the dividing cells show again subtetraploid or hyperploid numbers. Towards the end of the life span, the tumor cells suddenly decrease in number, and the degenerating cells are invisible in the hemorrhagic ascites. From the above findings, it is highly probable that the growth of the Watanabe ascites hepatoma is caused by the proliferation of the tumor cells constituting subdiploid, subtetraploid and subhexaploid populations.

Discussion

In the course of the cytological studies on the changes caused by long repeated subcutaneous injections of hot water (72°C) in rats, the senior author (F.W.) found a tumorous solid growth in the liver of an animal. The tumor thus produced was transformed into an ascites form by injecting the tumor pieces into the peritoneal cavity of rats. The histological observations the original tumor tissues and those after transplantation revealed that the tumor here concerned is a kind of atypical hepatoma. It is noteworthy that the wall of the cysticercus cyst formed the border between the original tumor tissue and the liver tissue, and that within the border the hepatic cells closely simulate neoplastic cells in shape,

being characterized by a nodular proliferation. This feature suggests the high possibility that the tumor cells originated from hepatic cells inside the cysticercus wall. Histological studies of the wall of cysticercus cysts of the rat liver by Watanabe and Urano (1954) lead to the conclusion that the present tumor developed from the hepatic cells inside the cysticercus wall. But, a question has remained unsolved whether the original tumor was directly induced by repeated injections of hot water or not.

Cytological observations of the present tumor, Watanabe ascites hepatoma showed the presence of at least three populations provided with subtetraploid, subdiploid and subhexaploid tumor cells respectively. The tumor cells of each population are characterized by the chromosome constitutions specific to each. Investigation of the behavior of the tumor cells made it clear that the growth of the tumor is attributable to the proliferation of these tumor cells.

Detailed study of the chromosomes of the present tumor is in progress. Further statement on the chromosomes must be postponed until the completion of that study.

SUMMARY

A new transplantable tumor was produced in the liver of a rat which had received repeated subcutaneous injections for many weeks with hot water at 72°C. The tumor tissue was crushed and injected in the peritoneal cavity of several rats; this treatment resulted in the production of an ascites tumor provided with freely suspended tumor cells.

After the histological observations of the tumor tissue it was found to be a sort of atypical hepatoma. It was proposed to designate this tumor as the "Watanabe ascites hepatoma."

The chromosome investigation of the tumor cells indicated that there are present at least three populations characterized each by subtetraploid, subdiploid and subhexaploid tumor cells. The growth of the tumor is primarily attributed to the proliferation of these tumor cells.

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Explanation of Plate VI

Fig. 1. Topographical view of the original tumor animal of the Watanabe hepatoma. l; liver t; tumor.

Fig. 2-4. Original tumor, haematoxylin and eosin staining.

Fig. 2. Tumor cells simulating the hepatic cells. Note basophilic small-sized cells intermingled with larger tumor cells. ×600.

Fig. 3. Showing large-sized tumor cells having multinuclei. ×600.

Fig. 4. Proliferation of hepatic cells simulating neoplastic cells, in the space between the tumor tissue and hepatic tissue. Tumor tissue in upper right, liver tissue at lower left; in the middle part is present the cysticercus wall. ×150.

Fig. 5. Tumor tissue at the 1st generation of subcutaneous transplantation, haematoxylin and eosin stain. Masses of tumor cells arrange themselves very separately. The cytoplasm increases in volume, the nuclear size remaining unchanged. ×150.

Explanation of Plate VII

Figs. 6-8. Chromosomes of the tumor cells of the Watanabe ascites hepatoma. Camera-lucida drawings, ca. ×1800. Fig. 6; subdiploid. Fig. 7; subtetraploid. Fig. 8; subhexaploid.

Figs. 9-11. Serial alignments of chromosomes of tumor cells. Fig. 9; subdiploid. Fig. 10; subtetraploid. Fig. 11; subhexaploid.

要　旨

渡辺腹水癌の一般的性状

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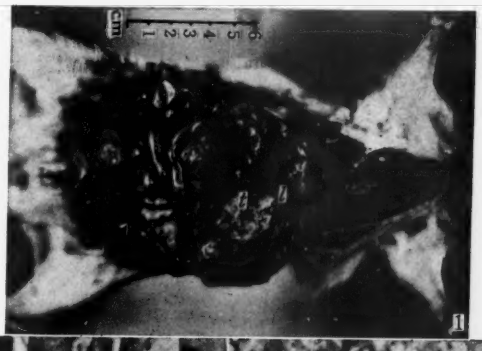
渡辺 (1954) は 72°C の熱水を連続皮下注射したシロネズミのうちの1頭の肝臓に結節状の腫瘍を見出した。この腫瘍の一部は皮下および腹腔内に移植された。その結果，腫瘍は結節性の皮下腫瘍と，腹水性の遊離細胞型としてそれぞれ増殖し，累代移植が成功した。

原発腫瘍および皮下移植腫瘍の組織学的研究によって，この新腫瘍は肝癌の一種であることが明らかとなり，腹水型の腫瘍を‘渡辺腹水癌’と命名した。

渡辺腹水癌は腫瘍の一般的性状ならびに細胞学的特性において，吉田肉腫，MTK 肉腫などとは著しく異っている。特に，腫瘍細胞の染色体研究より，現在の所この腫瘍には少くとも 2n-, 4n-, および 6n- 型の種族細胞が混在していることが明らかとなった。これら3つの種族細胞はそれぞれ特有の染色体構成を有し，腫瘍の増殖に主要な役割をなしている。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CYTOLOGICAL CONFIRMATION OF FLUID INFECTION IN THE VENEREAL TUMOR OF DOGS
(With Plate VIII and IX)

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INTRODUCTION

There are types of malignant tumors called ascites tumor that have their origin in the mutation of certain somatic cells; they are remarkable by their behaviour simulating that of monocellular parasitic organisms (Farden 1953). Extensive cytological studies of ascites tumors in rats by Makino and his coworkers (Makino 1952, Makino & Kanō 1953, 1955, Makino & Tonomura 1955, Makino & Tanaka 1953, Tonomura 1953, Yosida 1954, Makino & Tonomura 1955, *etc.*) revealed that in every tumor there are a population or populations of stem-cells with characteristic chromosome patterns, and that the proliferation of stem-cells is responsible for the growth of the tumor. Like monocellular organisms, these tumor cells grow in tissue fluid (*i.e.* lymph or ascites) or blood. Typical examples are represented by leukemias and ascites tumors; the tumor cells are freely suspended and proliferate in blood or ascites. The so-called fluid tumors such as leukemias and ascites tumors developing in rats or mice are capable of artificial transplantation from host to host by injection of tumor cells (Yoshida 1949). Because of this method of transplantation some authors have described these lesions as being ascites infection (Mckee et *al.* 1953).

If the malignant tumors such as leukemias or ascites tumors are capable of transmission like monocellular organisms, natural transmission of these tumors would be possible as occurs in infections caused by parasitic microorganisms or protozoa. The senior author (Watanabe 1953, 1955) suggested the possibility of natural transmission in a venereal tumor of dogs. In the present paper the results of some experiments undertaken to confirm natural infection in this interesting dog tumor are to be presented.

The authors take much pleasure in expressing their sincere gratitude to Professor Sajiro Makino of Hokkaido University for kind revision of the manuscrept for publication.

General Aspects of the Infectious Venereal Dog Tumor

The infectious venereal tumor of dogs generally develops in the external genitalia of mature dogs of both sexes. It is readily transmissible through copulation. During the past forty years many unsuccessful attempts have been made to demonstrate a pathogenic viral agent which is separable from living cells. Formerly the venereal tumor of dogs was regarded as an infectious granuloma by some authors (Weile 1919, Kingery 1921). Recently, Nanta and his coworkers considered the transmission process to be inflammatory in nature (1948). But many authors have agreed that the venereal tumor is consistently neoplasmic (Sticker 1906, Beebe and Ewing 1906, Hunter, Laws and Loeb, 1909, Stubb, Fruth 1924, Imamaki 1932, Bloom, Paff, Noback 1951, Karlson, Mann 1952). Some pathologists have classified the venereal tumor as a kind of carcinoma, while others have considered it as a reticulum cell, alveolar or a round cell sarcoma. Based on the nature of origin of this venereal tumor, several pathologists (Kaalund-Jrgensen and Thomsen 1937, Bloom, Paff and Noback 1951) have recommended using the term "transmissible venereal tumor".

In females, the tumor of the vaginal wall is manifested as granular, reddish nodules or submucosal mass; sometimes they attain hen's egg-size, and infiltrate into the external labien and the adjoining parts. The tumor usually increases in size following parturition and frequently tends to bleed, ulcerates and eventually undergoes necrosis. In males, there is a variation in the clinical picture. In the early stage, single or multiple small, reddish nodules are seen on the affected part of the penis; they infrequently grow larger than about 5 cm. in diameter and often infiltrate the scrotum. Generally sanguineous, serous and putrid secretions (or discharge) exude from the tumor surface. Metastasis of the tumor occurs rarely in regional lymph nodes and in some internal organs.

The venereal tumor of dogs is artificially transplantable and successive transfers are possible from dog to dog. The tumor sometimes shows a clinical tendecy of healing; this seems to indicate a high degree of immunity in the affected dog. It is important to know that the experimental transplantation of the tumor may be accomplished only by the use of fresh material containing viable tumor cells. The artificial transmission can also be demonstrated by rubbing the tumor cells into the scarified mucous membrane of the penis of the dogs (Stubbs & Furth 1934).

The experimental observations revealed that the growth of the neoplasm in the new animals that received transplantation of the tumor was due to proliferation of the transplanted tumor cells (Hunter, Law & Loeb 1909). This observation supports the belief that a viral agent is not a cause of the venereal tumor of dogs. Transmission of the tumor is also accomplished by means of copulation (Smith & Washbourn 1898). Microscopical observations indicated that the infectious venereal tumor of dogs is comprised of round, oval or polyhedral cells with indistinct boundaries and cytoplasm poorly staining with hematoxylin-eosin. There are no granules or inclusion bodies in the cytoplasm of tumor cells. The nuclei are large, and round or oval in shape: they are vesicular with minute clumps of chromatin and contain distinct nucleoli. Under low power magnification, the parenchymal cells of the tumor are generally uniform in size and shape, being supported by a delicate connective tissue stroma in earlier stages of development. The connective tissue fibers become broader in later stages showing an alveolar structure characteristic to the tumor tissue. Usually many mitotic figures of tumor cells are present in the growing tumor tissue. There are observable inflammatory cells and necrotic areas particularly in the partially ulcerated surface. In the hollows of the ulcerated surface of the tumor, there are many isolated cells or cells intermingled with

delicate fibers (Watanabe, 1954).

One of the important features of the venereal tumor of dogs is that the serous, hemorrhagic and purulent fluid contains a number of large round cells together with leucocytes, erythrocytes, micrococci and bacilli. This fluid usually stagnates in small, shallow areas on the tumor surface. Sometimes fluid drips from the tumor. This fluid contains a number of freely suspended tumor cells in the process of mitotic division (Watanabe 1955). Thirteen casses so far observed by the authors during the past several years showed the same features.

It is highly probable that cells suspended in the fluid from the venereal tumor of a dog are naturally transferred into the mucosa of the external genitalia of another dog during copulation. This possibility was first discussed in the literature by the senior author in 1955. To accomplish the natural transmission of the venereal tumor the constant occurrence of viable tumor cells and their continuous proliferation in the tissue fluid from the tumor are necessary. Cytological investigation of the tumor cells in the venereal tumor of dogs was undertaken to confirm the evidence of fluid infection. The results of this study are described in this paper. Thirteen dogs with similar venereal tumors have been studied. Details of findings on the tumor of dog No. 13 will be presented.

Material and Methods

Dog No. 13 was a seven years old female mongrel who has a history of five previous pregnancies. About one year ago, a thumb-tip-sized tumor was found in her external genitalia. The tumor was reddish in color. It gradually increased in size. It had an uneven surface of a cauliflower-like appearance (Figs. 1-3). Purulent and hemorrhagic fluid stagnated in several shallow depressions existing on the surface of the tumor. It grew into a hen's-egg-size and had a scar-like

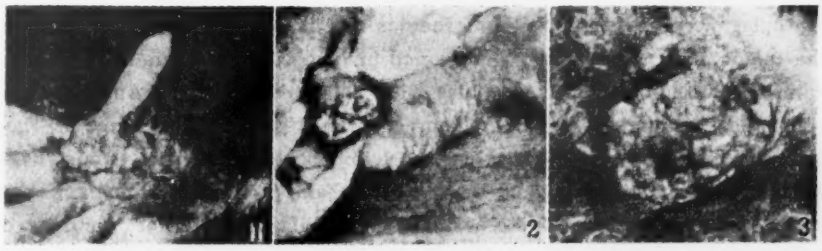

Fig. 1. Large mass of an ulcerated tumor infiltrating around the root of penis exudes serous fluid (No. 9). **Fig. 2.** Tumor developed from vaginal wall measures 4.0×2.5 cm. A large round tumor projected left-upwards, outside the infiltrated vaginal wall (No. 8). No. 8 and No. 9 are histologically round cell sarcomas. **Fig. 3.** Infectious venereal tumor on external genitalia of dog No. 13, 7 years old small female mongrel. A hen's-egg-sized tumor in compact mass measures 7.0×5.0×2.5 cm. On the remarkable uneven surface, hemorrhagic fluid containing many tumor cells, is always recognizable. No metastatic growth. It shows the structure of a round cell sarcoma.

appearance in its peripheral parts. No metastatic growth was found. When

touched with an instrument, the tumor became readily hemorrhagic and the dog seemed to feel a pain. Histological observations made it clear that this was a kind of a round-cell sarcoma. The hemorrhagic and serous fluid could be obtained at any time from the shallow depressions of the ulcerated tumor surface. For the histological study the soft tumor tissue was removed.

Methods: The tissue fluid found in the shallow depressions of the ulcerated tumor surface was transferred on slides with a fine pipette and stained with Giemsa's solution. The chromosome investigation was done on slides smeared with the tissue fluid and stained with acetic dahlia following water-treatment or non-treatment technique (Makino 1954).

Histological sections of tissue fixed in 10 percent formalin or Orth's solution were stained with hematoxylin-eosin or according to Bielschowsky's method.

Cytological Observations of Cells Occurring in the Tissue Fluid Stained with Giemsa's Solution

Considerable numbers of large and round tumor cells were intermingled with many leucocytes, saprophytic cocci and a small amount of bacilli. The tumor cells were freely separated or in groups consisting of several or sometimes many cells. It seemed apparent that the group formation of cells may accidental or by technical sequence, since there was no connection between the tumor cells (Fig. 7).

The tumor cells were generally uniform in size and shape, and characterized by a considerable amount of pale blue cytoplasm; sometimes there were projecting cytoplasmic processes. Neither granules nor inclusion bodies were observed in the tumor cells. The nuclei were round or oval being uniform in size. Each contained a nucleolus stained pale blue with Giemsa's solution. Apparently the morphological features of the free tumor cells were generally the same as those of parenchymal cells of the tumor tissue. Lymphocytes were rarely found; they resembled smaller tumor cells. Degenerated, faintly stained tumor cells with pyknotic, granular nuclei were occasionally noted. Damaged mitotic figures were rarely observed. The occurrence of tumor cells in process of mitotic division was frequent. Binucleate cells and tripolar mitoses were rare.

From the results of the above observations it is supposed that the proliferation of the free tumor cells has been going on in the fluid of the tumor.

Cytological Observations of Tumor Cells in the Smeal Preparations after Acetic Dahlia Technique

Smear preparations of tumor fluid showed many tumor cells interspersed with

a few leucocytes. They were round or sometimes ovoid and uniform in size (Fig. 8, Fig. 9). Frequent mitotic figures showing different stages of division were intermingled with degenerated tumor cells (Fig. 9). The general appearance seen in the tumor fluid prepared by acetic dahlia technique was similar to that found in the Giemsa preparations.

Histological Observations of the Venereal Tumor

Parenchymal cells of the tumor were round or oval in shape, rather uniform in size, but somewhat irregular (Fig. 4). The cells contained considerable amount of cytoplasm showing a weak affinity to eosin. They contained neither granules nor inclusion bodies. The nuclei were large and round or oval. Each nucleus included fine clumps of chromactin together with one or two well-defined nucleoli. Eosinophilic, mononuclear cells were frequent. Lymphoid cells of small size were occasionally observed. Capillaries and connective tissue were scanty. Fibroblasts were rather frequent here and there. Scattered silver stained fibers were found in the older tumor tissue (Fig. 5).

The results of the above histological observations indicate that the general structure of the venereal tumor of dog No. 13, was apparently the same as that of the round cell sarcoma. It is remarkable that mitotic figures in various stages of division were frequently observed in the tissue. Usually each microscopic high power field contained two or three such figures.

Mitotic Rate of the Free Tumor Cells in the Tissue Fluid

Based on three preparations containing many tumor cells, the number of cells in process of division and the mitotic rate were calculated after observing 2000 tumor cells in each preparation. The results are given in Table. 1.

Table 1. Number and frequency of mitotic figures, from 2000 tumor cells in three specimens, prepared from the fluid on the tumor.

Stage	Specimen		
	A	B	C
Prophase	12	13	12
Meta.	17	18	14
Ana.	1	1	2
Telo.	7	1	3
Total	37	33	31
%	1.85	1.65	1.55

The mitotic rate varied from 1.55 percent to 1.85 percent. The mitotic figures at metaphase appeared at the highest frequency. In the first preparation 17 metaphase figures occurred in 37 dividing cells; the second preparation showed 18 metaphase figures in 33 dividing cells, and the third contained 14 metaphase fegures in 31 dividing cells. In each of the three preparations, the number of mitotic figures decreased in the order of prophase, telophase and anaphase. It

is notable that the frequencies of mitotic cells as seen in the fluid of the dog venereal tumor showed a fair agreement with those found in the ascites tumor of rats, such as Yoshida sarcoma (Hirono 1954), and the MTK-sarcoma (Tonomura 1954). The division rate in the range of 1.55 to 1.85 percent is generally similar to that observable in the latter period of the life span of the ascites tumor-bearing rat.

Next, the frequencies of prophase, metaphase, anaphase and telophase were studied based on 1030 dividing cells (Table 2). There were 620 mitotic figures at metaphase (*i. e.*, 60.19 percent); they were largest in number in the material under study. The frequency decreased in the order of prophase, telophase and anaphase. The lowest frequency was observed in anaphase. The number of abnormal mitotic figure was 18 (*i. e.*, 1.75 percent). This included four multipolar mitoses, six cells with abnormal scattering of chromosomes, two cells with stickiness of chromosome and two binucleate cells.

The infectious venereal tumor of dog here concerned seems to show low malignancy, but the frequency of mitotic figures is considerably high especially in the growing part of the tumor.

Table 2. Number and frequency of regular mitotic cells and abnormal cells, based on the total number of 1030 dividing cells.

Stage	Number of cells observed			%
Prophase	251			24.37
Meta.	620			60.19
Ana.	41			3.98
Telo.	100			9.71
Abnormal mitoses	multipolar cell	4	18	1.75
	bi-nucleate cell	1		
	bi-nucleate cell bridge formation	1		
	scattering of chroms.	6		
	stickiness of chroms.	6		
Total	1030			100.00

Observations of the Chromosomes in the Tumor Cells

The number of chromosomes of the tumor cells in the tissue fluid were observed in several clear metaphase plates. The number varied from 40 to 67. The majority of the cells was found to have 46–64 chromosomes; the greatest frequencies were 54 to 56 per cell. Twenty eight cells contained 54 chromosomes in each (*i. e.*, 18.9 percent of the 148 mitotic figures observed), 21 cells contained 56 chromosomes in each, 12 cells 57 chromosomes and 14 cells 58 chromosomes (Table 4). The variations of the chromosome number seems to take place around the number 54, there being a range of fluctuation from about 40 to 67. Detailed investigations with sufficient material are needed before a conclusion can be made that the cells having 54 chromosomes are the stemline-cells in this infectious venereal tumor of the dog.

In the acetic dahlia preparations the chromosome complex was analysed in some detail in well-preserved six metaphase plates. Table 5 shows the chromosome numbers and complexes observed in these six cells. As seen in Table 5, the chromosome complex consists in each of certain numbers of rod-shaped elements in addition to V-shaped ones which vary from 2 to 4 in number. The metaphase examples are shown in Figures 13 to 20.

According to Minouchi (1928) and Makino (1952), the diploid number of the dog is 78, consisting of 77 rod-shaped elements and a V-shaped one which is the X chromosome. In comparison with the chromosomes of the ordinary cells of the dog, the chromosome complex of the venereal tumor cell is remarkable in showing a rather decreased number of rodshaped chromosomes together with an increased number of the V-shaped ones.

Table 3. Number and frequency of dividing cells, based on the total number of 2000 tumor tissue cells.

Stage	Specimen		
	A	B	C
Prophase	10	15	18
Meta.	14	17	16
Ana.	1	0	3
Telo.	0	0	2
Total	25	32	29
%	1.25	1.60	1.45

Table 4. Variation of chromosome number ranging from 40 to 67, the modal number being 54.

Chromosome numbers	40	42	43	44	45	46	48	49	50	51	52	53	54	55	56	57	58	60	61	62	64	66	67
Number of cell observed	1	1	1	1	1	5	7	1	7	2	1		28	6	21	12	14	7	3	5	3	1	1

Table 5. Chromosome numbers and constitutions in six tumor cells

Chrom. no.	Chrom. complex
52	22 R's + 28 r's + 2 V's
54	22 R's + 28 r's + 4 V's
54	22 R's + 28 r's + 4 V's
56	24 R's + 28 r's + 4 V's
56	26 R's + 26 r's + 4 V's
60	31 R's + 26 r's + 3 V's

Discussion

One of the biological interests of the venereal tumor of dogs is that the tumor is readily transmissible from dog to dog through copulation, and also is capable

of artificial transmission by the use of the material from the tumor containing viable tumor cells. All attempts to transfer the tumor by inoculating tumor filtrate have failed. No microbial or pathogenic viable agent has ever been demonstrated previously. Smith and Washbourn (1898) seem to be the first to show the transfer of the venereal tumor of the dog by means of copulation. Based on the results of his experiments, Imamaki (1932) concluded that the tumor cells derived from the venereal tumor could be directly transferred into the injured mucosa of the external genitalia during copulation. His explanation seems to be supported by the experiment that the transplantation of the tumor is accomplished by rubbing the tumor cells into the scalified mucous membrane of the penis of the dog.

For the transmission of the venereal tumor of the dog by copulation there are two necessary factors. One is that many proliferating tumor cells must be suspended in the hemorrhagic or serous tissue fluid. The other is the injuries of the mucous membrane of the external genitalia which make the inoculation of tumor cells possible during prolonged contact of the diseased and healthy genitalia by copulation. The latter seems to be supplemented by the fact that the venereal tumor occur very frequently in the concurrently conceived dogs (Smith & Washbourn 1898). The results of the present investigations reveal that the morphological features of the freely suspended tumor cells in the tissue fluid are the same in every respect as those of the parenchymal cells of the venereal tumor, and that the tumor cells continue to proliferate.

The incidence of mitotic figures ranges from 1.55 to 1.85 percent. The mitotic rate thus obtained approaches that observed in the latter part of the life span of the rat which bears ascites tumor. The low mitotic rate may probably be due to the secondary disturbance by some microorganisms in the fluid, since there are many cocci and bacilli in the fluid.

The frequency of metaphase figures in tumor cells observed in the fluid of the dog venereal tumor is found to be the highest. The frequencies show a decrease in order of prophase, telophase and anaphase. It is interesting that the frequency value obtained in the present tumor tends to agree with that observed in the ascites tumor of the rat (Hirono 1954, Tonomura 1954).

It is shown that the number of chromosomes of the tumor cells of the present venereal tumor has a wide range of variation from 40 to 67. So far as the scope of the present study is concerned, the tumor cells containing 54 chromosomes appears at the highest frequency (18.9 percent). It is premature to conclude that the cells having 54 chromosomes constitute the stemline of the present tumor, since the material is insufficient to decide the situation at present. The frequency value of chromosomes per cell decreases around the "54-cell". The

chromosome complex was analysed in some detail in six tumor cells as seen in Table 5. It was found that the chromosome complexes of the tumor cells consist of certain numbers of rod-shaped elements in addition to V-shaped ones varying from 2 to 4 in number. The diploid complement of the dog has been found to have 78 chromosoms consisting of 77 rod-type elements and a single V-shaped one which is the X-element (Minouchi 1928, Makino 1952). After comparison with the chromosomes of the ordinary cells, it is evident that the tumor cells here concerned are characterized by a rather reduced number of rod-shaped chromosomes in addition to an unusual number of V-shaped elements varying 2 to 4. Though the conclusion can not be made at present as to the cells which constitute the stemline of the present tumor, it is notable that the tumor cells invariably show a higher number of V-shaped chromosomes than in the ordinary cells. Makino (1952), Makino and Kano (1953, 1955), and Tonomura (1954) have demonstrated in various kinds of rat ascites tumors defiiite numbers of the distinct large V-shaped chromosomes which are absent in the ordinary cells characterzing the type of the tumor. Similar feature occurs in some mouse tumors (Hauschka 1953). As shown in the above descriptions, the venereal tumor of dogs here concerned also contains in excess distinct V-elements. The existence of V-shaped chromosomes in the tumor cells is thus remarkable in different kinds of tumors, and it is of significance in considering the origin and development of the tumor in general.

In summary, the present investigations made it evident that the venereal tumor of dogs is capable of natural and artificial transfer through the application of the fluid from the tumor surface. It was found further that the tissue fluid contained a number of proliferating tumor cells in suspension, and that the transmission of the tumor is a result of the presence of these active tumor cells. After transmission the tumor cells proliferate through successive divisions and contribute to the formation of the tumor in the new host. It is interesting that the tumor cells show fewer chromosomes. There is reduced number of rod-shaped elements; V-shaped ones are increased, numbering 2 to 4.

Summary

The cytological investigations of the free tumor cells occurring in the fluid of the venereal tumor of a dog were undertaken to confirm the evidence of so-called "fluid infection" in the dog venereal tumor.

1) The investigated venereal tumor of a dog has a cauliflowerlike appearance. Purulent or hemorrhogic tumor fluid stagnates in shallow depressions of the tumor.

2) Histological observations of tumor tissue made it clear that this is a kind of the round-cell sarcomas.

3) In the fluid from the tumor surface, many round tumor cells are observed that are generally the same as the parenchymal cells of the tumor tissue.

4) Mitotic figures of the suspended tumor cells in various stages of division are frequent.

5) The number of chromosomes of the tumor cells in the fluid shows a range of variation from 40 to 67, with the peak incidence at 54 to 56. In six well preserved metaphase plates, it was found that each cell contains variable numbers of rod-shaped elements and additional 2 to 4 V-shaped ones.

6) The results of the present study reveal that the fluid from the surface of the dog venereal tumor contains a number of actively dividing tumor cells in the form of suspension, and the natural transfer of the tumor is due to the application of the fluid containing tumor cells to the injured genital mucosa.

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Explanation of Plate VIII

Fig. 4, many round or ovoidal cells with a few mitotic figures, being surrounded by connective tissue. From dog No. 13. 600.
Fig. 5, distinct fibers give an alveolar structure. X 600.
Fig. 6, several mitotic figures found in immature tumor tissue. X 1000.
Fig. 7, round tumor cells intermingled with leucocytes in the fluid. Giemsa's stain. X 600.
Fig. 8, two mitotic figures found in tumor cells, from a smear preparation Acetic dahia squash technique. X 600.
Fig. 10, a tumor cell at metaphase, showing 54 chromosomes: V-shaped ones are observed Water-treated, acetic dahlia squash technique. X 1500.
Fig. 11, irregular scattering of chromosomes at metaphase. X 1500.
Fig. 12, cells at telophase. X 1500.

Explanation of Plate IX

All are camera-lucida drawings. Figs. 13–18, 19–20: ca. X 2000.
Figs. 13–18, metaphase plates of tumor cells. From dog No. 13.
Fig. 13, metaphase plate of a tumor cell having 54 chromosomes. Four elements are V-shaped, in each.
Fig. 14, metaphase plate showing 54 chromosomes.
Fig. 15, metaphase plate showing 56 chromosomes. Four elemuts are V-shaped in each.
Fig. 16, metaphase plate showing 58 chromosomes.
Fig. 17, metaphase plate showing 60 chromosomes, 3 elements being V-shaped.
Fig. 18, metaphase plate showing 66 chromosomes.
Figs. 19–20, serial alignments of chromosomes in tumor cells.
Fig. 19, chromosome number, 54. 22 elements are rod-shaped, 28 rod-or granular-shaped, and 4 are V-shaped.
Fig. 20, chromosome number, 60. 31 chromosomes are rod-shaped. 26 rod-or granular-shaped, and 3 are V-shaped.

要　旨

液状伝染に関する犬伝染性外陰部腫瘍の細胞学的研究

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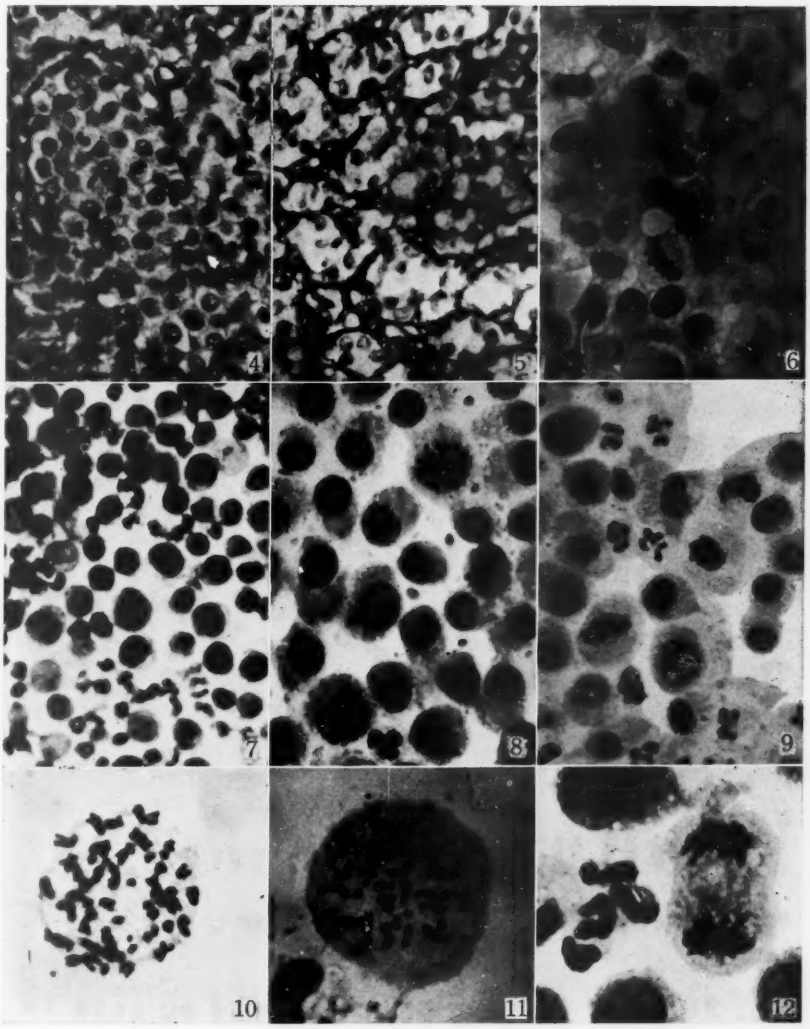
(長崎大学医学部家畜医学研究所)

体細胞に由来する悪性腫瘍が宿主内で独立して生活し，組織液内に浸潤増殖する一方血行あるいは淋巴道によって転移を起す状況は顕微鏡的単細胞生物の繁殖形式に似ているものといえる。特にマウスやラットの血液あるいは腹水中に瀰漫性に浮游増殖する白血病あるいは腹水腫瘍の細胞が営む行動は，生体内における微生物のそれと類似するものがある。かくのごとく腫瘍細胞が単細胞生物のごとく行動し繁殖しているものとするならば，細菌あるいは原虫のごとき単細胞生物が示す伝染という現象が，特殊な状況においてはかかる腫瘍の間にも行われ得るものとの想像が許される。すなわち，生物学的な立ち場から腫瘍をみるときに，腫瘍がその宿主内において自己の種族を繁殖させるとともに，何等かの過程によって他の宿主に移行し，そこで増殖して同じ腫瘍を繁殖させる行動が考慮される。

かかる見解に立って，ウィールスその他の病原体が証明されないところの犬の伝染性外陰部腫瘍をとりあげて，その伝染性の成り立ちにつき細胞学的考察を試みた。最近長崎地方において観察した 13 例の犬の伝染性外陰部腫瘍は組織学的にはいづれも円形細胞肉腫に近い像を示していたが，全例共に外陰部の腫瘍の潰瘍表面に腫瘍細胞を多数含む白濁液が絶えず滞留あるいは滴下していることが特異であった。この一見液状の腫瘍を思はせる分泌液内の腫瘍細胞が，交尾という機会に際し，犬に特有な痙攣的且持続的な交尾形式によって恐らく機械的に障害された反対性の性器粘膜下に自然移植され，そこに腫瘍が発生して腫瘍細胞の自然移植が成立するものとの見解に達した。これが確定にはかかる腫瘍細胞を含む分泌液による移植実験が必要であることはいうまでもないが，この液を介して腫瘍が自然移植される過程を腫瘍の液状伝染という言葉で表現した。これは腫瘍細胞が液状腫瘍の形態をとって他の宿主に自然に移行する現象を意味する。犬外陰部腫瘍分泌液による液状伝染が成立するためには分泌液内で多くの腫瘍細胞が健全に生活し，しかも分裂増殖していることが必要条件となってくる。

最近発見した雌小犬の外陰部腫瘍分泌液内の腫瘍細胞につき細胞学的観察を行った結果は，一定の核学的構成の存在を暗示し，染色体数 54 を持つ細胞が高い頻度において出現する。この細胞学的観察の結果は，この犬外陰部腫瘍が交尾という機会において液状伝染の方法によって個体から個体に伝搬されることを有力に示すものである。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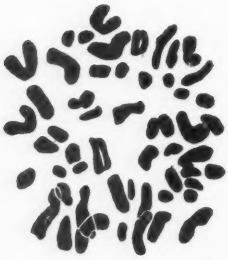

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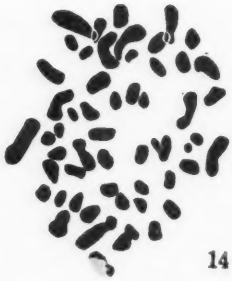

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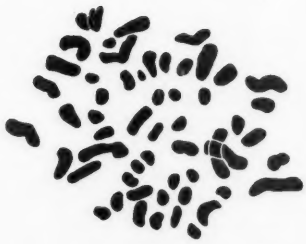

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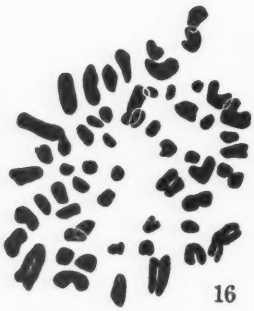

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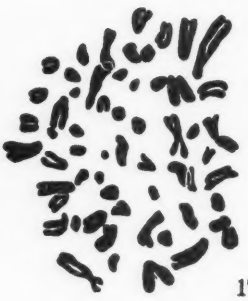

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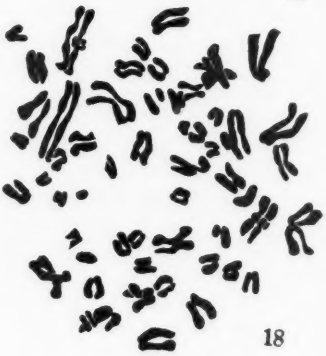

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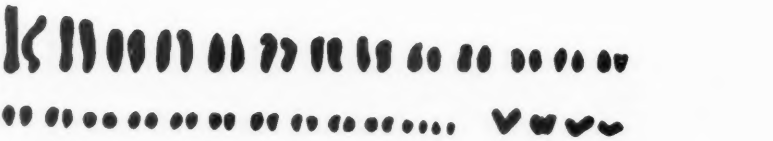

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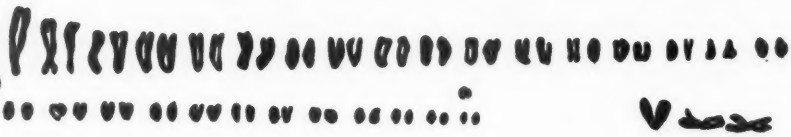

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[GANN, 47, 37~49; March, 1956]

INHIBITORY EFFECT OF THE HIGH MILIEU UPON THE DEVELOPMENT OF THE EXPERIMENTAL LIVER CANCER

(With Plates X—XIV)

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The results of research in this laboratory for these past years tell for affirmative that the frequency of tumor incidence in man is subject to geographical factors concerning its distribution, being rather high in the lower, dampf terrains and always rarer in the high level,[1-7] and that the same seems to be roughly valid for the frequency of the spontaneous chicken tumors.[8] Thus, these facts seem to suggest in themselves that there may exist a correlation between development of certain tumors and the life milieu of the host, especially the natural milieu. Notwithstanding, there have hardly been reported experimental data attacking this point hitherto. The authors have occupied themselves, as a link of the high clima study of this University, with series of the transplantable chicken sarcoma and found its rate of growth less favored at the high altitude than in the district of lower level.[9] Further, they succeeded in establishing the validity of the analogical principle concerning the experimental induction of liver cancer with p-dimethylaminoazobenzene (DAB) over a time span of 150 experimental days, and the research was extended to the histopathological findings in the liver, pituitary, the adrenal glands and also the hematopoietic organs.

METHOD

As the location of experiment, the Institute of Altitude Medicine of Nagoya University on Mt. Norikura, Japan Alps, was selected. This mountain is about 2,800m above the sea level, with 549.4mm Hg atomospheric pressure, of atmospheric temperature 11.4°C, and of 83.7 per cent humidity (averages for August, 1954). The work ensued in the Institute from July to the end of September; then it moved to State Cosmic Laboratory, Tokyo University, lying near on the same mountain and was continued until late in December under regulated air condition of 10°C. As the material served 49 rats of 70-80 gr body weight and of mixed

inbreed (high level group). Linked with this, a parallel experiment was carried on in our laboratory in Nagoya, with 757.7 mm Hg atomospheric pressure, of atomospheric temperature 27.2°C, of 76.8 per cent humidity (averages for August, 1954), as control with 36 rats of the same status and the same breed (low level group). The animals of both series were fed with a basic diet of unpolished rice containing DAB in a ratio of 0.06 per cent beside necessary amount of vegetables. Care was taken as to let the test animals receive the total amount of about 450mg DAB per head; the carcinogen was administered for 123 days to the high level group and for 120 days to the low level one. Thereafter both groups were fed with the above mentioned diet without DAB for subsequent 27 or 30 days. The comparison of the rate of liver cancer development was made on the 120 th and 150 th day after the commencement by killing a certain number of rats. On the other hand, the liver, all lobes of which were subjected to careful examination, the pituitary, the adrenals and the bone marrow were taken out each on the 7th, 14th, 120th and 150th experimental day, fixed in 10 per cent formalin and stained routinely with hematoxylin-eosin for histological study. Examination of the peripheral blood took place each separately on the 10th and 42th day in each group.

Results

As the general sanitary survey tells, there were 16 mortality cases among 49 samples in the high level group and 2 cases among 36 samples in the low level one up to the end of 4 months. Then until the close of experiment, there was none in the former group and 2 in the latter. The cause of death was mainly due to respiratory infections except one in the low level group that succumbed to liver cancer with extensive metastasis. Body weight increased by 30 to 50 g for the high level group and by 70 to 90 g for the low level one, toward the 4 th to 5 th month respectively.

The rate of the liver cancer development in the 4th and 5th months under consideration of the marcoscopical liver finding:—

The rat liver of the high level group on the 120th experimental day weighed 3.6 to 9.7 g, was brownish to darkish of color and presented typical aspects of nodular cirrhosis (Fig. 1, 3, 4, 5). The cancerous growth, though still indistinct to naked eyes, was established in 4 out of 9 examined cases positively. Therefore, tumor incidence stands here at 44.4 per cent. The control liver samples of the low level group weighed 7.7 to 15.0 g and presented uneven surface of whitish to yellowish taint with indistinct annular structure (Fig. 1,6). Cancer incidence of this group was established macroscopically in one sample and histologically

in 4, thus making 62.5 per cent tumor incidence. The liver of both groups on the 150 th day, looking not much unlike to the afore-mentioned material in exterior (Fig. 2), gave tumor incidence of 55.5 per cent for the high level group, with 3.

Table 1. Response of DAB-fed rats maintained on high and low altitude at 4th month

	high level group	low level group
Number of cases (sex)	9 (♂3, ♀6)	8 (♂4, ♀4)
Survival after commencement	16 / 39	24 / 26
Average body weight	93.3	128.1
Average liver ratio with body weight	7.3	8.3
Tumor formation		
macroscopical	0	1
microscopical	4	4
Total	4	5

Table 2. Response of DAB-fed rats maintained on high and low altitude at 5th month

	high level group	low level group
Number of cases (sex)	9(♂4, ♀5)	14 (♂5, ♀9)
Survival after 4 months	9 / 9	14 / 16
Average body weight	115	149
Average liver ratio with body weight	7.7	8.1
Tumor formation		
macroscopical	3	4
microscopical	2	9
Total	5	13

macroscopical and 2 microscopical instances among 9 samples; the incidence was 92.9 per cent for the low level group, with 4 macroscopical and 9 microscopical instances among 14 cases. Tables 1 and 2 give review of samples used, survival, average body weight, average liver ratio and frequency of tumor formation; these data indicate that the high altitude milieu has inhibitory effect upon the development of the experimental liver cancer.

Histological Findings of the Liver

As a survey of the literature on the administration of DAB indicates,[10-15] histological findings of the liver during the course of experiment, so manifold as they are, could purposefully be set apart into the benign and malingant changes; the former again into cirrhosis, nodular hyperplasia, cystic ducts and cholangiofibrosis. Of these, the cirrhosis, a never-failing accompaniment alongside the growth process of the experimental liver cancer, tends to develop intensely beneath

the capsule but always weak toward the center of the love. Under "mild form" of cirrhosis one understands sheer proliferation of the fibrous tissue around the portal tract (Fig. 7), under "moderate form" the pseudolobule formation in slight degree (Fig. 8) and under "nodular form" the annular structure all over the entire lobes (Fig. 9); lastly, such compression form of lobules all around through exuberant proliferation of fibrous tissue will be named "islet-like" (Fig. 10). Three major precancerous changes, i. e., nodular hyperplasia (Fig. 11), cystic ducts (Fig. 12) and cholangiofibrosis (Fig. 10), shall again be subdivided each into

Table 3. Histological liver changes in DAB-fed rats maintained on high and low altitude at 4 months

	high level group	low level group
Types of tumors		
Hepatoma	6	8
Cholangioma	0	1
Mixed	0	1
Anaplastic	0	0
Total	6	10
Cirrhosis		
mild	0	0
moderate	0	1
nodular	9	6
islet-like	0	1
Total	9 / 9	8 / 8
Nodular hyperplasia		
mild	2	2
extensive	7	2
Total	9 / 9	4 / 8
Cholangiofibrosis		
mild	3	4
extensive	0	3
Total	3 / 9	7 / 8
Cystic ducts		
mild	4	3
extensive	1	0
Total	5 / 9	3 / 8

Table 4. Histological liver changes in DAB-fed rats maintained on high and low altitude at 5 months

	high level group	low level group
Types of tumors		
Hepatoma	2	12
Cholangioma	1	5
Mixed	8	3
Anaplastic	0	1
Total	11	21
Cirrhosis		
mild	0	6
moderate	0	1
nodular	9	4
islet-like	0	3
Total	9 / 9	14 / 14
Nodular hyperplasia		
mild	5	4
extensive	3	2
Total	8	6 / 14
Cholangiofibrosis		
mild	1	5
extensive	1	5
Total	2 / 9	10 / 14
Cystic ducts		
mild	1	3
extensive	2	1
Total	3 / 9	4 / 14

a mild and an extensive form. The nomenclatures of neoplasm, hepatoma (Fig. 13, 14), cholangioma, (Fig. 15) mixed type (Fig. 16) and anaplastic type (Fig. 17) were adopted, roughly in accordance to predominant histological feature. And, in the present experiment, all the neoplasms belonged to carcinoma, and none to sarcoma. It must be noted that the very beginning of the cancer development excepted, a tumor made out of one single type belongs to a rare happening, and one often meet with concomitance of every tumor type as well as transitional figures within them.

While Table 3 and 4 include the data concerning the malignant changes noticed in all liver lobes, those treating the benign ones are tabulated for convenience sake from the data obtained from median lobe (Lobs. 3 and 4). Though changes in each liver lobe are likely to tend to render comparison with one another easy, Lobs. 1 and 6 may sometimes be affected with certain attenuation of changes such as cholangiofibrosis (low level group) or nodular hyperplasia (high level group), contrasted with the intermediated stand taken by median lobe and others.

Findings of 5 high level samples (3 males and 2 females) and 5 low level samples (3 males and 2 females) on the 7th experimental day presented no remarkable deviation nor difference between the groups. Whereas, the hepatic cells of the high level samples (3 males and 2 females) of the 14 th day revealed a marked degeneratiou in the periphery of lobules by lack of proliferative process therein, the low level samples (3 males and 2 females) displayed certain proliferation of bile duct cells.

On the 120 th and 150 th days, hepatic findings of the high level group present features so far different from those of the low level group as, firstly, the cirrhosis of this group is bound up by fibrous tissue typically annularly as to constitute pseudolobuli of various sizes and, tangibly reminding Laennec's type in man; there is indistinct inflammatory cell infiltration and proliferation of bile duct (Fig. 18). The fibrous area around portal tracts, much vascular and hyperemic of capillary blood vessels, are also prominent (Fig. 19). In good contrast the cirrhosis of the low level sample reveales a right indiscriminate annular structure and there are to be found in interstitium the sign of inflammation as well as bile duct proliferation always in abundance.

Secondly, cholangiofibrosis designated by Opie, which produces irregular, map-like structure of hepatic lobules, closely associated with above mentioned "islet-like" type of cirrhosis, may often cover the most of liver substance especially in Lobs. 1 and 6 in the low level samples (Fig. 10). On the contrary it is met with in the high level samples only scantily.

Thirdly, although extensive nodular hyperplasia is more frequently to be seen in the high level group than in the low level one (Table 3, 4) at the same time, a great variety of degenerative structural changes of this type is also often encountered in the former though rare in the latter, the retrogressive enlargement of nuclei and cytoplasm which may progress into karyolysis and cytolysis (Fig. 20) or pycnosis of nuclei and hydropic vacuolation of cytoplasm (Fig. 21).

As the rare, fourth criterion of the high level specimen there may take place now disseminated, focal necrosis, histologically representing a sort of coagulation necrosis affecting liver cells or cells in nodular hyperplasia. It was established in 7 out of 8 120 days' cases and may unite together to a big heap with one

another (Fig. 22). Under the circumstance, there may occur a unique change in some of such heaps thus distinguishing zone of central necrosis, zone of leucocytic infiltration and zone of peripheral hemorrhage (Fig. 23). Certainly this is a novel finding with reference to DAB-induced liver cancer experiment, and may have some bearing on the high altitude milieu. By the way, it should not be passed unoticed that after 27 days' cessation of DAB administration, necrotic fields just mentioned were missed almost entirely in the high level samples, suggesting the participation of the drug in the induction of foregoing necrotic lesions.

As is to be seen in Tables 3 and 4, though there is a trifle difference in the histological types of tumors between both groups in 120 days' samples, it becomes more diversified in 150 days' samples; for instance, the hepatoma type comes by 57.1 per cent in the low level group and by 18.2 per cent in the high level one, the cholangioma type by 23.8 per cent in the former and by 9.1 per cent in the latter, while the mixed type makes 72.6 per cent of the high level group and 14.3 per cent of the low level group. Really, pure cholangioma type was found in 5 cases of the low level samples, whereas there was only one to be seen in the high level ones.

Histopathology of Pituitary and Adrenal Glands

Tissues were taken from material of 7 th, 14 th, 120 th and 150 th day. In the earlier stages, there is certain reduction of alpha cell type paralleling the increase and hypertrophy of beta cell type in the anterior lobe; adrenal cells are subjected to individual manifoldness of change ranging from hypertrophy to deterioration. After all, the changes come always more marked in the high level group. In tissues of the high level series and of later dates as 120 days and longer, the anterior lobe cells become much shrunk and are provided with pycnotic nuclei, and there is marked degenerative change in the posterior lobe cells accompanying abnormal dilation of blood vessels (Fig. 24), whereas the adrenal cortex appears by now much thinner with its cells again atrophied. In good contrast, the anterior lobe of the low level samples is seen bearing an inclination toward proliferative hypertrophy of beta cell at the cost of alpha cell (Fig. 25). In cells of zona reticularis of adrenal cortex, there prevails again proliferative hypertrophy, even leading to adenomatous hyperplasia eventually, while cells of the adrenal marrow tend to fall very readily to hyperplasia, deterioration or even to vacuolation, in both high and low level groups.

All in all, one thing is ascertainable that the above changes in both organs, plainly subject to individual difference but common to both level groups, surely stand in parallelism with one another, thus suggesting an intimate relation exist-

ing between the two organs.

Histopathology of the Bone Marrow and the Blood Finding

Bone marrow preparations of 5 cases of both level groups taken on the 7 th, 14 th, 120 th and 150 th days and those of the peripheral blood of equal numbers taken on the 10 th and 42 th days were examined on blood cell count, hemoglobin (per cent) and blood picture, preparations of the peripheral blood of 5 rats from the low level stock and of DAB-free feeding serving as normal control.

In the high level samples, there were noticed indications of accentuation of erythropoiesis such as enlargement of the marrow sinusoids and hyperplasia of erythroblast contrasted to much reduced leucopoiesis accompanied by increase of reticulocyte, whereas material coming from the low level group characterises

Table 5. Blood cell count of peripheral blood.

	Exp. days	Leucocyte	Erythrocyte ($X10^3$)	Hemoglobin (%)
High level	10	8230(7600–9600)	7010 (6080–7840)	88 (75–103)
Group	42	5880(3600–6700)	6970 (6100–8060)	99 (95–104)
Low level	10	9160(6500–14400)	4360 (3330–5690)	73 (67–82)
Group	42	14320(13400–15700)	5820 (4750–7010)	82 (78–90)
Normal		12520(8300–17400)	6600 (6020–7530)	85 (80–90)

Averge of 5 cases for each of data.

itself through accentuation of leucopoiesis. Linked with these findings as demonstrated in Table 5, cell count of peripheral blood of the high level samples revealed increase of erythrocytes with leucopenia and that of the low level ones returned leucocytosis. Polychromasia came in both groups, but more pronounced in the high level samples. This reversal in leucopoiesis influenced by alitude factor seems to stand in connection with varied intensity of inflammatory process in the liver according to prolonged stay at each altitude, high and low.

Discussion

To prepare proper food has been the vital point in experimental production of the liver cancer as it has been thought that, in case of using azo-dye as the carcinogen, the factors inhibiting production of the tumor must lie in the combination of diet. From the fact that the liver cancer abounds among the Orientals, the noted rice-consumers, one thing has been elucidated that, between essentials of the nutritive agents working inhibitory upon the natural liver cancer of man and that influencing the experimental one, there must lie a discrepancy of pretty

wide range. The present work has been started purporting to verify the relationship between the life milieu of an individual, especially natural milieu, and carcinogenesis. And the result tells in affirmative that the experimental carcinogenesis through DAB becomes more inhibited in the high altitude milieu than in lower terrain.

Before considering essential of this factor, one better think over things specific to the high altitude, for example, the low oxygen pressure of the locality measuring at the neighborhood of 549.4 mm Hg. Still, one would hardly reconcile with the idea and adopt unanimously the condition as the vital factor.

Some time ago, Iwase and others[16] studied organs of rat long maintained on high altitude and succeeded in establishing remarkable alterations in the anterior lobe of pituitary and the adrenal cortex beside those in the bone marrow. From this, one would be led inevitably to the concept of general-adaptation-syndrome of Selye[17] based on the functional link system of pituitary-adrenal cortex.

Many investigators have noted that the pituitary exerts a direct effect on carcinogenesis and also on the growth of tumors. Recently, the fact has been brought into light by Griffin and others[18,19] that hypophysectomy impairs formation of liver tumors in rats fed azo-dye. Further, Symeonidis et al.[20] found that adrenalectomized rats without adrenal cortical regeneration, fed DAB and treated or not treated with desoxylcorticosterone acetate, do not develop liver tumors. These speak in positive for the important role played by the pituitary or adrenals of the host against the deadly action of the carcinogenic substance. Perhaps there is no lack of by-proofs, as Richardson et al,[21] proved, through the use of methylcholanthrene, cell atrophy or degeneration of adrenal cortex beside inhibitive effect on development of experimental liver cancer, and Symeonidis et al.[20] could, through other means, bring forth degenerative atrophy of adrenal cortex, also. Degenerative atrophic process traced in the anterior lobe of pituitary, and adrenal cortex of the high level group over a time span of 120–150 days would probably serve as a fitting pathfinder along this line of study, although sufficient data are not presented in this report.

Summary

Purporting to settle experimentally the problem of carcinogenesis in connection with the life milieu, especially the natural milieu, p-dimethylaminoazobenzene was administered to rat up to 450 mg per head in average, 4 to 5 months long and in groups where one group was maintained at the altitude of about 2800 m and atmospheric pressure of 549.4 mm Hg and the other group on a low terrain. The results are summarized as follows:

1) Tumor incidence of the test animal gave 44.4 per cent with the high level group against 62.5 per cent with the low level group toward the 120th experimental days and 55.5 per cent with the former against 92.9 per cent with the latter toward the 150th experimental day. This denotes that carcinogenesis in the liver caused through DAB suffers inhibition in the high altitude than in the lower level. Histological features of the liver cancer somewhat differ from each other according to the living altitude.

2) Hepatic changes of benign nature and of DAB origin projected a vertical difference to two level groups against each time extent of 4 to 5 months. In the high level group, the affected area was rich in nodular cirrhosis with a distinct annular texture but poor in cholangiofibrosis; on the contrary, in the low level group, the annular structure was less pronounced among cirrhotic area against well developed cholangiofibrosis accompanied by inflammatory cell infiltration. Besides, a variety of degeneration of nodular hyperplasia cells is encountered more in high than in low level samples through 4 to 5 months, and a peculiar liver necrosis was noticed in the high level samples at the 4th month (which seemed effaced with disuse of DAB).

3) Comparing the histological findings of hormonal organs each at the 4th and 5th month, there was established degenerative atrophy of the cells of anterior lobe of pituitary and adrenal cortex in the high level group and some hypertrophy plus hyperplasia of the cells of these organs in the low level group. Certain consideration was paid on the correlation between carcinogenesis and the functional changes of these hormonal organs.

4) There were established also erythropoiesis cum leucopenia in the high level group and leucopoiesis in the low level group.

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Explantation of Plates X—XIV

Fig. 1. Livers from rats fed DAB of both high and low level group at 4th month. Left 2 rows (9 samples) indicate those of high level group and others (8 samples) those of low level group.

Fig. 2. Livers from rats fed DAB of both high and low level group at 5 th month. Left 2 rows (9 samples) indicate those of high level group and others (12 samples) those of low level group.

Figs. 3, 4 and 5. Livers of high level group reveal macroscopically distinct annular texture.

Fig. 6. The liver of low level group reveals macroscopically indistinct annular texture.

Fig. 7. "Mild" type of cirrhosis (high level group, 4 th month).

Fig. 8. "Moderate" type of cirrhosis (high level group, 4 th month).

Fig. 9. "Nodular" type of cirrhosis (high level group, 4 th month).

Fig. 10. "Islet-like" formation of liver lobules associated with cholangiofibrosis (low level group, 5 th month).

Fig. 11. Nodular hyperplasia (high level group, 4 th month).

Fig. 12. Cystic ducts (low level group, 5 th month).

Fig. 13. Hepatoma of adenocarcinoma type (high level group, 5 th month).

Fig. 14. Hepatoma of solide type (high level group, 5 th month).

Fig. 15. Cholangioma type (low level group, 5 th month).

Fig. 16. Mixed type in which transitional figure ot hepatoma and cholangioma is observed (low level group, 5 th month).

Fig. 17. Anaplastic type of liver cancer (low level group, 5 th month).
In this case, the transitional figure from cholangioma type was noticed.

Fig. 18. The fibrous tissue around large portal tract with scanty formation of bile duct proliferation and weak inflammation (high level group, 4 th month).

Fig. 19. Increase and hyperemia of capillaries in fibrous tissue are prominent (high level group, 4 th month).

Fig. 20. Degenerative swelling of cells of nodular hyperplasia (high level group, 4 th month).

Fig. 21. Degenerative type of nodular hyperplasia which reveals pycnosis of nuclei and vacuolation of cytoplasm (high level group, 5 th month).

Fig. 22. Focal necrosis of the liver (high level group, 4 th month).

Fig. 23. High power view of Fig. 21 with central necrosis, leucocytic infiltration, aud peripheral hemorrhage.

Fig. 24. Pituitary atrophy in high level group at 4 th month.

Fig. 25. Hyperplasia of beta cell type of pituitary in the low level group at 4 th month. Note the same power magnification in Fig. 24.

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要　旨

高地環境における実験的肝癌の抑制

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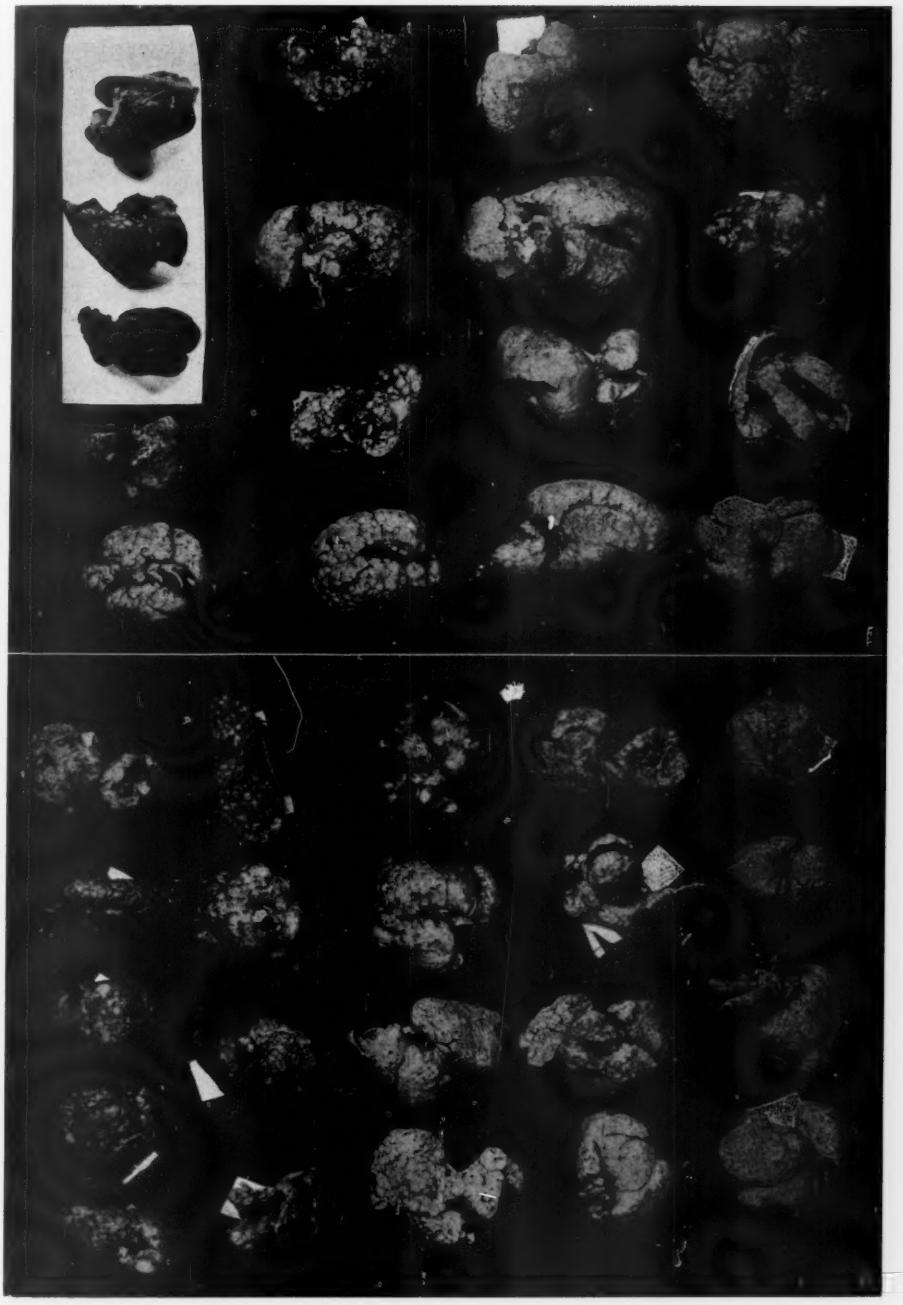
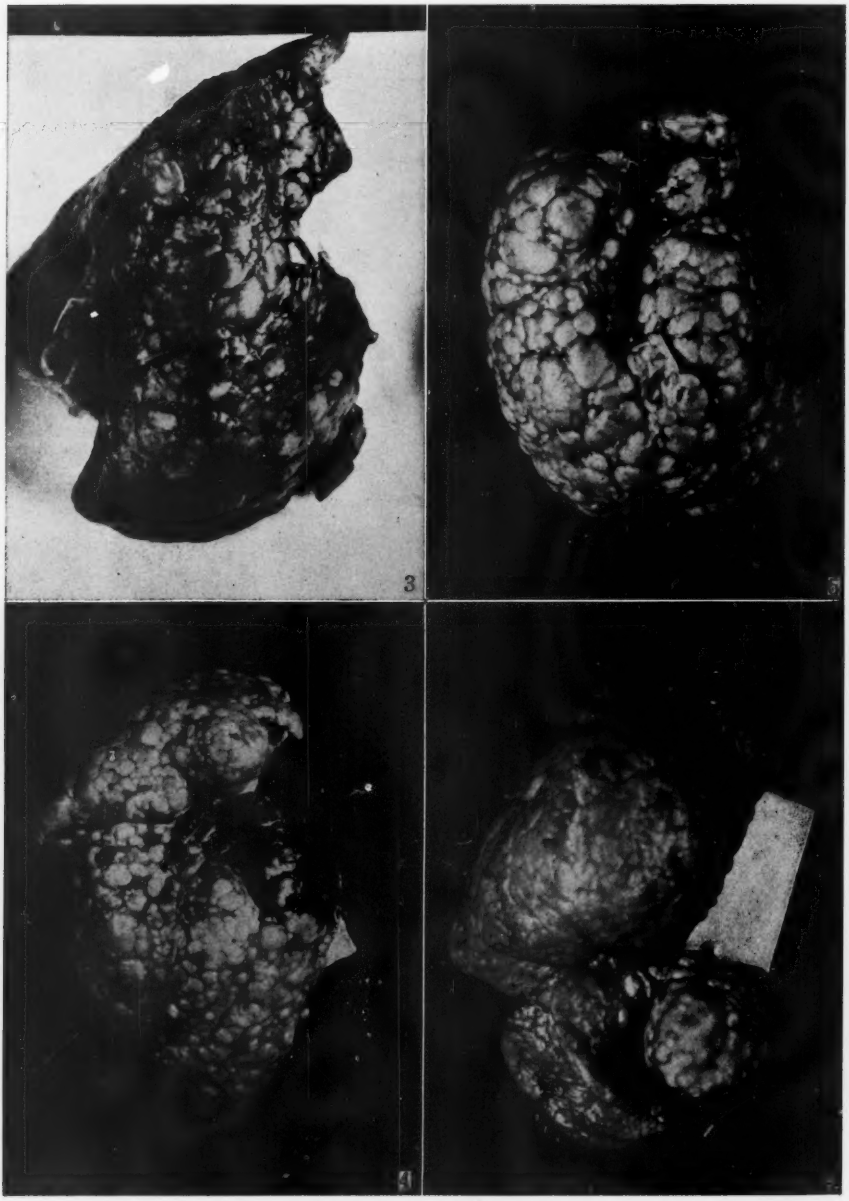
腫瘍の発生と個体の生活環境殊に自然環境との関係を実験的に追究するために，4～5ヶ月に亘つて高地及び低地でラッテを飼育し，p-dimethylaminoazobenzene を投与し，両者の癌発生の状態を比較した。この結果高地 (日本アルプス乗鞍岳，標高約 2800 m，1954 年 8 月の平均気圧 549.4 mmHg，気温 11.4°C，湿度 83.7%) に飼育した動物の肝癌発生は同一条件で低地 (名古屋，同年同月の平均気圧 757.7 mmHg，気温 27.2°C，湿度 76.8%) に飼育したそれより抑制されることが明らかになった。すなわち実験動物の肝癌発生率は実験 4 及び 5 ヶ月において高地群ではそれぞれ 44.4% 及び 55.5% であるに対し，低地群ではそれぞれ 62.5% 及び 92.9% であった。さらに前癌性の組織変化において両群の間にかなりの差異があり，高地群では明瞭な輪状肝硬変像を示すこと多く，低地群では cholangiofibrosis の像強く一般に輪状構造は不明瞭である。又高地群では結節性肥大の細胞の変性像強く，また 4 ヶ月では特異な肝細胞壊死像を多数例に認めた。

わが教室の多年に亘る地理病理学的研究により，人間の悪性腫瘍の発生が低湿の地に多く高地に少ない事実が明らかにされたが，以上の成績は癌の発生並びに発癌過程と個体の生活環境殊に自然環境との間に密接な関係のあり得ることを実験的に明示したものである。

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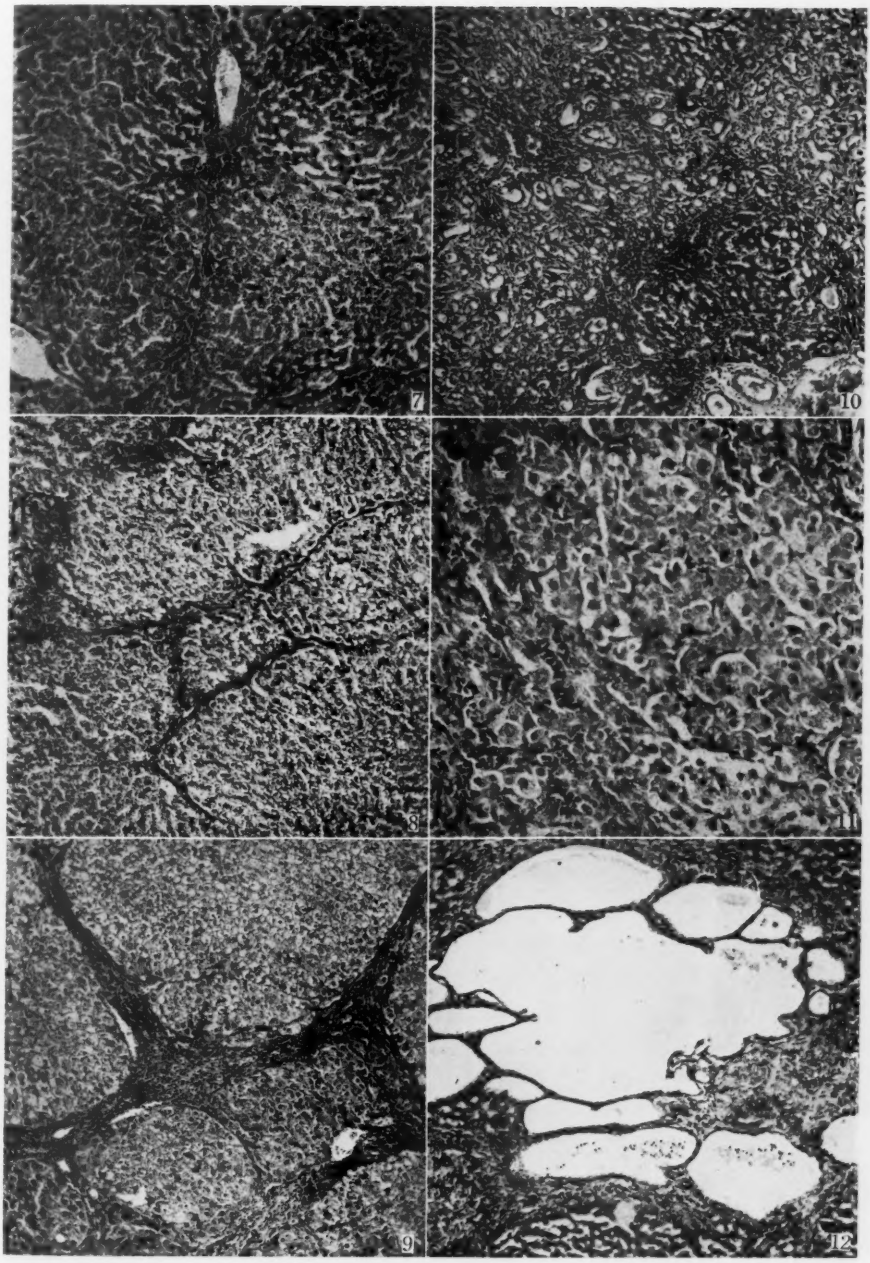
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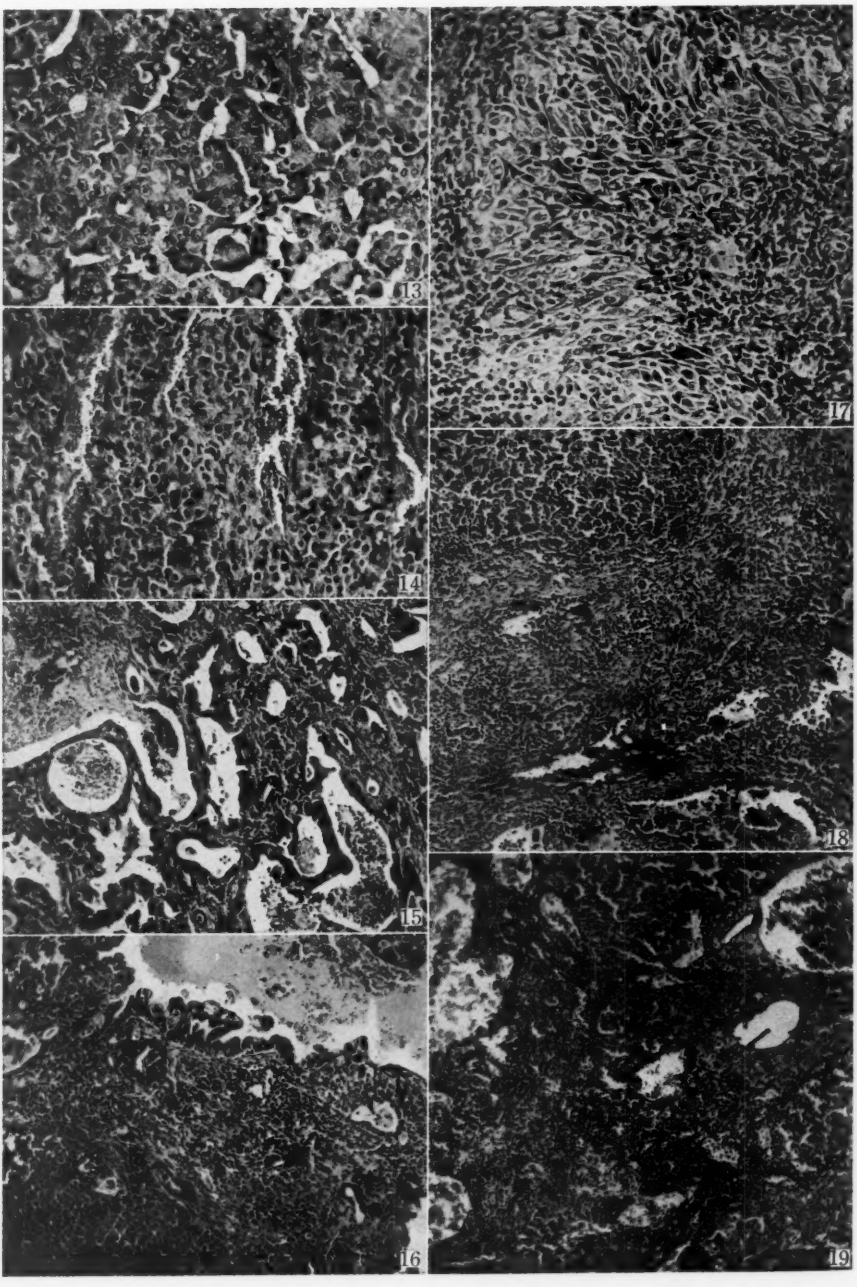
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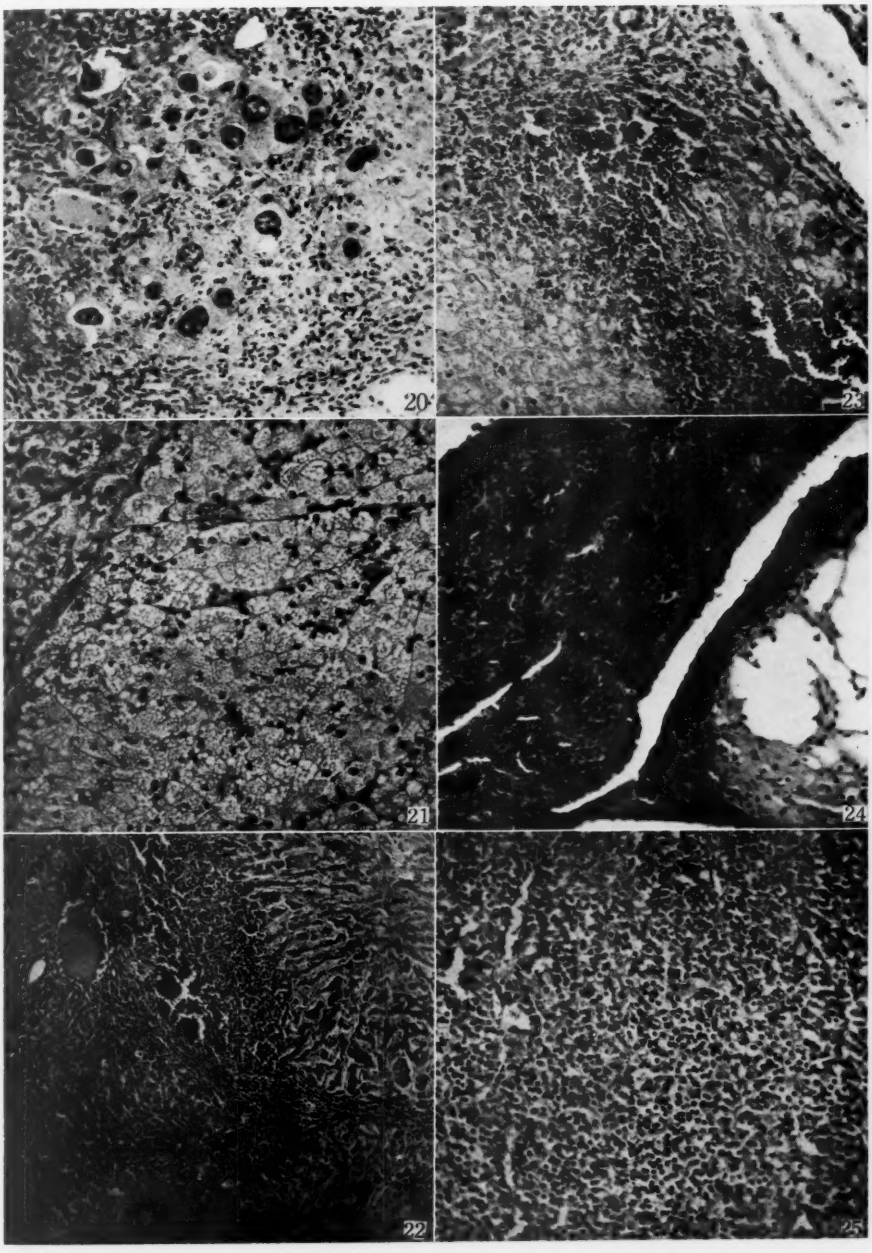
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[GANN, 47, 51~78; March, 1956]

EXPERIMENTAL STUDY OF XANTHENE DYES AS CARCINOGENIC AGENTS (With Plates XV-XVII)

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INTRODUCTION

For the past several years I have been engaged in the studies on the carcinogenicity of xanthene dye stuffs, and since the first demonstration in 1952 (1) of sarcoma production by rhodamine B injections, similar experiments were carried out using many kinds of the dyes belonging to the same group. It is the purpose of this paper to bring together all these experimental results and to describe them in full.

Xanthene dyes, because of their utilization in artificial coloring of food articles, etc., have been investigated in the past as to their possible carcinogenic action. Yao (2), and later Okamoto, Furukawa and Kawaji (3) fed rhodamine B mixed (0.1-0.2 per cent of the rice) with the usual laboratory diet to rats for over 200 days (Total rhodamine B: about 2.04 g), but they failed to find any notable pathological changes either in the liver or in other organs.

In 1953 Willheim and Ivy (4) also reported negative results with xanthene dyes (erythrosine, eosine, pyronine, phloxine and rhodamine B). The dyes were fed mixed with ground "Purina Fox Chow" at the rate of 4 per cent. Feeding experiments for 18 months to two years gave only negative results.

In spite of the negative results of these feeding experiments it seems premature definitely to conclude that these dyes are non-carcinogenic, for different methods of administering the dyes might give quite different results. In this connection, the method of repeated subcutaneous injections suggested itself as the one that should be tried.

It may be recalled that Nishiyama (5) produced sarcoma experimentally by repeated subcutaneous administeration of concentrated glucose. Later, not only glucose (Takizawa (6), Nonaka (7), Ito (8), Sugishita (9) and Arakawa (10)), but glycogen (Amano (11), Ito (8)), fructose (Takizawa (12)), galactose (Takizawa (13)), and lactose (Arakawa (10)) were also found to evoke sarcomas under similar experimental conditions.

In 1937 Schiller (14), and in 1947 Harris's (15) reported that sarcomas were pro-

duced by repeated subcutaneous injections of 1 to 3 cc of 2 per cent watery solution of light green SF once or twice weekly. Also, in 1949 J. Gillman, T. Gillman and Gilbert(16) reported that trypan blue was carcinogenic for rats. In this experiment, rats received fortnightly subcutaneous injections of 1 cc of aqueous solution of trypan blue, and developed reticulum cell tumors of the liver in periods of betwee 150 and 300 days.

In view of these reports I have undertaken the subcutaneous injections of xanthene dye stuff (rhodamine B, rhodamine 6 G, fluorescein sodium, eosin G, rhodamine 3 G, fluorescein, erythrosine and violamine R), and it was by this method that I succeeded in producing sarcoma in rats at the site of injections of certain of these dyes.

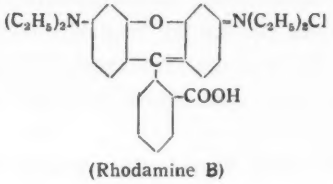
In the present paper are first given details of the injection experiments in which certain dyes gave positive sarcoma production (Exps. I-V), and certain others failed to produce sarcoma (Exps. VI-IX).

Some attempts were made to modify the course of the sarcoma production by the combined use of other substances with the carcinogenic xanthene dyes, and accounts of these experiments are next taken up (Exps. X and XI). Feeding experiment with negative result is referred to as confirmatory data for the work of previous authors (Exp. XII).

Finally, negative experiments using mice are briefly described (Exps. XIII-XV). It is of interest to note that xanthene dyes which are productive of sarcoma in rats, proved to be inactive for mice not only when fed but also when repeatedly injected.

Sarcoma Production by Injections of Rhodamine B, Rhodamine 6 G, Fluorescein Sodium or Eosine Yellowish

Experiment I. In this experiment, the effect of rhodamine B injections was examined. Rhodamine B is a kind of xanthene dye stuff, a hydrochloride of diethyl-m-amino-phenolphthalein, or tetraethyldiamino-o-carboxy-phenyl-xanthenyl chloride (Colour Index No. 749; $C_{28}H_{31}N_2O_3Cl$), forming green crystals, the watery solution of which has rose color with a strong fluorescence. The substance is said to be used in Japan for coloring various food articles and for the preparation of rouge, theatrical make-up and toilet-powder.



(Rhodamine B)

Experiment was started with 20 normal adult albino rats of a mixed Saitama strain, all weighing around 150 g. They were kept in wire cages in groups of two each, and were maintained on the usual laboratory diet of whole wheat with occasional supply of dried fish, vitamins A and D concentrate, green vegetables and dried yeast.

The preparation of rhodamine B used in the experiment was a product of the Tokushu Chemical Co., Ltd., Tokyo. The rats were given subcutaneous injections, at as nearly the same site as possible on the back, of 1 cc of 0.5 gdl. watery solution of rhodamine B two to three times a week. The concentration of the solution was increased to 0.75 gdl after 1.5 months, to 1 gdl after 3 months, and to 1.5 gdl after 9 months. After about one year of repeated injections, when the rats tended to became weak, the concentration of the solution was gradually decreased. During this stage of the experiment, injections were discontinued for a period of about 1.5 months, being resumed again after that period of interruption. Rhodamine B solution was sterilized by heating before injection.

Of the 20 rats, with which the experiment was started, 10 rats died early without showing tumor. Out of the remaining 10, which lived 420 days or more, receiving 80 injections during the period, 6 rats produced sarcoma at the site of the injections. (Table 1)

Table 1. Experiment I.

Rat No.	Sex	Exp. days	Rhodamine B		Body weight (end)	Tumor size (cm)	Histological diagnosis
			Total (mg)	Inject. No.			
16	f	450	900	87	/	2.5×3.0×1.8	Fibroblastic and spindle cell sarcoma
9	m	481	980	93	184	No tumor	
1	m	485	930	89	331	7.0×6.8×6.5	Fibrosarcoma
8	m	505	980	93	/	6.5×5.0×3.0	Fibrosarcoma
15	f	523	1060	101	209	1.8×1.8×1.4	Fibrosarcoma
7	m	532	1040	99	252	5.0×4.5×2.0	Fibrosarcoma
14	f	613	1155	115	192	No tumor	
17	f	652	1165	117	/	4.0×3.0×1.0	Fibrosarcoma
13	f	714	1275	132	/	No tumor	
18	f	750	2005	141	135	No tumor	

Individual records of these 6 rats are as follows:

No. 16. On the 420th day a hard nodule of the size of the large pea was palpated, which grew to the size of 2.5×3.0×1.8 cm. On the 433rd day. Animal died on the 450th day. Metastases were found in right axillary and right inguinal lymph nodes.

No. 1. A hard nodule of the size of 1.5×2.0×1.3 cm, first palpated on the 433rd day, grew to the size of 5.0×4.5×3.0 cm on the 462nd day. The animal died on the 485th day with the tumor measuring 7.0×6.8×6.5 cm; no metastasis.

No. 8. A hard nodule measuring 1.0×3.0×0.5 cm was palpated on the 462nd day. The animal died on the 505th day with the tumor measuring 6.5×5.0×3.0 cm; no metastasis.

No. 15. A hard nodule of the size of 1.8×1.8×1.4 cm was palpated on the 466th day. The animal died on the 523rd day, with metastasis in the left axillary lymph node.

No. 7. On the 513rd day the tumor measuring 5.0×4.5×2.0 cm was found. The animal was found very weak on the 532nd day and was killed; no metastasis.

No. 17. A hard nodule of the size of the small finger-tip was palpated on the 533rd day, which attained the size of 4.0×3.0×1.0 cm by the 631st day. The animal died on the 652nd day.

In these 6 rats, subcutaneous connective tissue became thickened and small hard consolidations of various sizes became palpable during the 14th to 18th month. These consolidations gradually turned into irregular nodules, which, then, rapidly grew into large tumors. The tumor grew very rapidly to a large size, and soon killed the animal.

All these tumors were found in the subcutaneous tissue at the site of the injections of rhodamine B, and were of the same general characters. They have smooth surface, sometimes with an ulcer formation with evident border on the circumference, and are closely adherent to the skin and muscular tissue. They are elastically hard, the cut surface generally pearly white, occasionally with pinkish or reddish areas due to haemorrhage, with hardly recognizable necrotic areas. There was no evidence of the imbibition of the dye in the tumor tissue.

No significat change was noted in internal organs at autopsy. No change in the nature of liver surface or color was recognized in gross and no tumor or cirrhosis was observed. Spleen showed hemosiderosis and fibrosis.

Histologically all the tumors are diagnosed as fibrosarcoma, being composed chiefly of fibrinoplastic spindle cells, showing mitotic figures frequently. The lymph node metastases found in the two rats coincided with the primary tumors in every histological feature.

Attempts to transplant the tumor to normal rats were made in the cases of No. 16, No. 15 and No. 7. The transplantation from No. 16 took in 100% of the animals in the first generation, 22% in the second, 20% in the third, 80% in the fourth, 30% in the fifth, 60% in the sixth, 50% in the seventh, etc. It is now in the 49th transplantation generation (100% takes). The transplantation from No. 7 took in 80% of the animals in the first generation, 67% in the second, 70% in the

third, 80% in the fourth, 78% in the fifth, 88% in the sixth, 89% in the seventh, etc. It is now in the 78th transplantation generation (100% takes).

In the case of No. 15, tumor tissue for transplantation was taken from animals that has been dead, and probably for this reason transplantation did not succeed.

The subcutaneous transplant develops in a nodular form which attains often to a hens egg or even to an india-rubber ball size in three or four weeks, and the rats die of tumors in about one month on an average after transplantation.

Experiment II. The above experiment was repeated in order to confirm the result.

Experiment was started with 30 normal rats (Wistar strain descendants), all weighing around 150 g, containing 17 males and 13 females. They were maintained on the same type of laboratory diet as before, and the sample of rhodamine B used was also the same. The rats were given subcutaneous injections, at as nearly the same site as possible on the back, of 1 cc of 0.5 gdl watery solution of rhodamine B two to three times a week. The concentration of the solution was increased to 0.75 gdl after 2 months, and to 1 gdl after 5 months.

In the course of the experiment 19 rats died early without showing tumor at the site of the injections, but one of them, dying on the 293rd day, showed hypertrophy of thymus (thumb-tip size). Out of the remaining 11, which lived 436 days or more, receiving 100 injections during the period, 2 rats produced sarcoma at the site of the injections. (Table 2)

Table 2. Experiment II.

Rat No.	Sex	Exp. days	Rhodamine B		Body weight		Tumor size (cm)	Histological diagnosis
			Total (mg)	Inject. No.	beginning	end		
1	f	450	820	97	157	136	No tumor	
2	m	457	915	109	150	190	No tumor	
3	m	462	925	110	157	160	No tumor	
4	m	476	900	104	151	249	7.0×6.0×4.0	Spindle cell fibrosarcoma
5	m	481	910	105	173	150	No tumor	
6	f	545	890	104	177	183	No tumor	
7	f	563	900	105	152	170	No tumor	
8	m	576	900	105	168	177	4.2×4.0×2.3	Spindle cell sarcoma
9	f	638	950	110	162	130	No tumor	
10	f	688	1060	120	180	197	No tumor	
11	f	765	1110	126	165	138	No tumor	

Individual records of these 2 rats are as follows:

No. 4. A nodule measuring 2.0×1.9×0.8 cm was palpated on the 436th day,

which attained the size of 7.0×6.0×4.0 cm by the 476th day, when the animal was killed in very weakened condition.

No. 8. A hard nodule of the size of small pea was palpated on the 259th day, which attained the size of the small finger-tip by the 503rd day. The animal died on the 576th day with the tumor measuring 4.2×4.0×2.3 cm.

No. 6, No. 7 and No. 9 in the table showed hypertrophy of thymus (small finger-tip or thumb-tip size). These as well as other cases of thymus hypertrophy found in rat dying earlier, may be regarded as of the spontaneous origin.

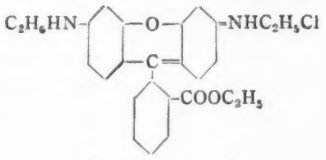
These tumors in Rats Nos. 4 and 8 were found in the subcutaneous tissue at the site of the injection. In general character they were much the same as those in Experiment I. No macroscopic metastasis was found in either of the cases.

These tumors were histologically diagnosed as spindle cell sarcoma, much the same as those in Experiment I. The tumor in Rat No. 4 showed more central necrosis than is usual for this type of tumors.

The liver was generally atrophic and histologically showed some hyperaemia and increase of Kupffer's stellate cells. Spleen showed sclerotic atrophy.

Attempt to transplant the tumor to normal rats was made in the case of No. 4. The transplantation took in 20% of the animals in the first generation, but did not succeed in the second.

Experiment III. Effect of injections of rhodamine 6G is next tested. Rhodamine 6 G is a kind of xanthene dye stuff, ethyl-ester of diethyldiamino-o-carboxy-phenyl-xanthenyl chloride (Colour Index No. 752 ; $C_{28}H_{27}N_2O_3Cl$), forming vermilion powder, the watery solution of which has scarlet-red color with a greenish fluorescence. The substance is said to be used for staining foodstuff, pomade, soap etc.



(Rhodamine 6 G)

Experiment was started with 16 normal rats of a mixed strain from the Saitama Prefecture, all weighing around 200 g. They were maintained on the usual laboratory diet of whole wheat with occasional supply of dried fish, cod-liver oil, rape-seed oil, dried yeast (Ebios), and green vegetables as in other experiments.

The preparation of rhodamine 6 G used in the experiment was the product of the Tokushu Chemical Co., Ltd., Tokyo.

Injections were made subcutaneously at as nearly the same site as possible on the back, in doses of 1 cc of 20 mgdl distilled water solution of rhodamine 6G two to three times a week. After about 4 months of repeated injections, when the

rats showed injury on the back, injections were discontinued for a period of about 1 month, being resumed again after that period of interruption. Rhodamine 6G solution was sterilized by heating before injection.

Of the 16 rats, with which the experiment was started, 7 rats survived the injection period of 487 days, receiving 100 injections during the period. Sarcoma developed in 4 of these rats (Table 3).

Table 3. Experiment III.

Rat No.	Sex	Exp. Days	Rhodamine 6G		Body Weight (end)	Tumor size (cm)	Histological diagnosis of tumor
			Total (mg)	Inject. No.			
1	m	502	21.6	103	/	No tumor	
2	f	511	20.8	99	158	2.9×2.8×1.6	Spindle cell sarcoma
3	f	531	22.2	106	137	1.9×0.8×0.2	Spindle cell fibrosarcoma
4	m	570	22.2	106	205	2.8×2.7×1.8	Spindle cell fibrosarcoma
5	m	609	23.6	113	147	No tumor	
6	f	628	23.6	113	233	5.0×4.1×1.5	Spindle cell fibrosarcoma
7	f	645	23.6	113	182	No tumor	

As many as 9 of the 16 rats died within 13 months after the begining of the injections without showing tumor. Rat No. 2 in the table, dying on the 511st day was the first to show tumor at the site of the injections. In this rat there was also an atrophic salivary gland with fibrosis. Rat No. 7, without the local tumor, was killed on the 645th day. It had a cysticercus sarcoma (spindle cell sarcoma) of the liver and a mammary fibroadenoma, which may be regarded as of the spontaneous origin, not connected with the experimental effect.

No. 2. A nodule measuring 2.3×1.7×0.5 cm was palpated on the 487th day. The animal died on the 511st day with the tumor measuring 2.9×2.8×1.6 cm.

No. 3. On the 531st day a hard nodule of the size of 1.9×0.8×0.2 cm was found. The animal was killed in very weakened condition on that day.

No. 4. A hard nodule of the size of the small-finger tip, first palpated on the 496th day, grew to the size of 2.1×1.5×0.9 cm on the 540th day. The animal died on the 570th day with the tumor measuring 2.8×2.7×1.8 cm.

No. 6. The tumor was found as a hard nodule of the size of 1.2×1.2×0.8 cm on the 557th day. The tumor reached the size of 2.7×2.3×1.3 cm on the 579th day. On the 628th day the animal was found in very poor physical condition and was killed.

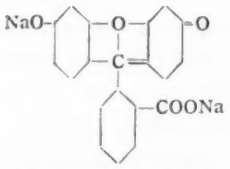
All these tumors were found subcutaneously, covered with a thin, white capsule,

not coalescing with any other surrounding tissues. They were elastically hard, cut surface evenly pearly white with hardly recognizable necrotic or hemorrhagic areas. There was no evidence of the imbibition of the dye in the tumor tissue. No macroscopic metastasis was found in any of the cases. No significant change was noted in internal organs at autopsy. The liver showed general atrophy and hyperaemia. No Kupffer's cells hypertrophy. No change in the nature of liver surface, color, or consistency was recognized in gross and no cirrhotic change was recognized histologically. Spleen showed hemosiderosis and atrophy. However, all these changes may be regarded as of no special significance in connection with the local sarcoma production.

All tumors are diagnosed as fibrosarcoma, being composed chiefly of fibrinoplastic spindle cells. Mitoses were numerous in most tumors, and in only a few were giant cells at all common, although nearly all tumors contained some. These giant cells have mostly the oblong or irregularly round shape.

Sarcoma of Rat 2 and of Rat 6 were successfully transplanted to normal rats of the same mixed strain. The first tumor gave the positive transplantation rate of 40% in the first generation, and the second tumor, 80%. Further transplantation was not made.

Experiment IV. Experiment using fluorescein sodium was next carried out. Fluorescein sodium (uranine) (Colour Index No. 766; $C_{20}H_{10}O_5Na_2$) is also a kind of xanthene dye stuff, a sodium salt of hydroxy-o-carboxy-phenyl-fluorone, forming yellowish-brown powder, the watery solution of which has yellowish-orange color with a yellowish-green fluorescence. The substance is said to be used mainly for the production of the eosine dyes; also to a limited extent in printing wool; also for tracing the course of water drainage in locating the source of contamination of wells.



(Fluorescein Sodium)

The experiment was begun with 15 albino rats of a mixed strain (Saitama strain), all weighing around 200 g. They were maintained on the usual laboratory diet.

The preparation of fluorescein sodium used in the experiment was the product of the Takeda Chemical Co., Ltd., Tokyo. Injections were made subcutaneously on the back of the rats as nearly the same site as possible every time. The injection of 1 cc of 5 gdl distilled water solution of fluorescein sodium was repeated

once or twice weekly as a rule. Fluorescein sodium solution was sterilized by heating before injection.

In the course of the experiment 7 rats died early without showing tumor at the site of the injections, but one of them, killed on the 171st day, had fibroadenoma of the mammary gland (1.2×0.9×1.0 cm) which may be regarded as spontaneous tumor. Out of the remaining 8, which lived 245 days or more, receiving 57 injections during the period, 2 rats (Nos. 1 and 6) produced sarcoma at the site of the injections. There were two other rats (Nos. 3 and 5) with small fibroma at the site of injection.

Table 4. Experiment IV.

Rat No.	Sex	Exp. days	Fluorescein Sodium		Body weight (end)	Tumor size (cm)	Histological diagnosis of tumor
			Total (g)	Inject. No.			
1	f	248	3.0	57	137	2.7×2.0×1.7	Polymorphous cell sarcoma
2	m	265	3.1	59	/	No tumor	
3	m	288	3.3	63	247	1.9×1.3×1.0	Fibroma
4	m	304	3.45	66	190	No tumor	
5	m	438	3.8	78	/	2.2×1.8×0.8	Fibroma
6	m	466	4.2	81	230	4.8×3.6×2.5	Polymorphous cell sarcoma
7	f	555	4.7	91	/	No tumor	
8	f	661	5.35	104	142	No tumor	

Pertinent data on these 4 rats may be seen from Table 4.

Rat No. 6 in the table, killed on the 466th day with tumor at the site of the injections, also had a cysticercus sarcoma (spindle cell sarcoma) of the liver. Also, Rat No. 7 dying on the 555th day, had fibrotic mammary glands with congestion (2.0×1.3×1.1 cm). These tumors may be regarded as of the spontaneous origin, not connected with the local sarcoma production, and they are disregarded in this table.

No. 1. The tumor was found as a hard nodule of the size of thumb-tip on the 239th day. The animal died on the 248th day with the tumor measuring 2.7×2.0 ×1.7 cm.

No. 6. A hard nodule of the size of small pea was palpated on the 301st day, which attain the size of the small finger-tip by the 445th day. On the 466th day the animal was found in very poor physical condition and was killed.

Macroscopic appearance of the local tissue exposed to the repeated injection was similar in this experiment to the preceding experiment of rhodamine B. Soon after an injection the eyes of rat were colored yellow, but as the dye was eliminated through the kidneys, their color returned to normal after 48 hours.

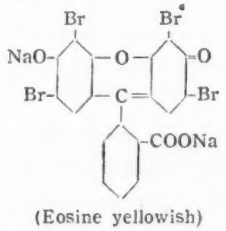
However, the subcutaneous tissues about the site of injection soon became deeply stained, some yellow-brown color persisted for a long time. The kidneys, too, became deeply pigmented. In the two rats (Nos. 1 and 6), subcutaneous connective tissue then became thickened and hard consolidations of various sizes became palpable with yellow-brown pigment. These consolidations gradually turned into irregular nodules, which, then, rapidly grew into large tumors. The tumor grew very rapidly to a large size, and soon killed the animal.

These tumors were found in the subcutaneous tissue at the site of the injection. In general character they were much the same as rhodamine B sarcoma (Experiment I), but showed no marked necrosis. There was no evidence of the imbibition of the dye in the tumor tissue. No macroscopic metastasis was found in either of the two cases. No significant change was noted in internal organs at autopsy. Spleen showed hemosiderosis.

The tumors were classed as polymorph cellular sarcoma with giant cells. Mitoses were numerous in most tumors. The tumors showed partly gelatinous degeneration.

Sarcoma of Rat 1 was successfully transplanted to normal rats of the same mixed strain. The first tumor gave the positive transplantation rate of 60% in the first generation, 78% in the second, 100% in the third, 78% in the fourth, and 50% in the fifth generation. Further transplantation was not made. These transplanted tumors attained an india-rubber ball size in about one month, and the rats died of tumors. Microscopically, the transplanted tumors were less polymorphic than the original tumor and classed as fibrosarcoma.

Experiment V. Another xanthene dye, eosine yellowish (eosin G), is tried in this experiment. This dye (Colour Index No. 768; $C_{20}H_6O_5Br_4Na_2$) forms red crystals, the watery solution of which has yellow-red color with a yellow-green fluorescence. The substance is said to be used in Japan for coloring various food articles, for nail polish powder and theatrical make-up, and for the preparation of rouge and of red writing and stamping ink.



(Eosine yellowish)

The experiment was begun with 20 albino rats of a mixed strain (Saitama strain), all weighing around 200 g. They were maintained on the usual laboratory diet of whole wheat with occasional supply of dried fish and green vegetables.

The preparation of eosine yellowish used in the experiment was the highest purity product (Merck). Injections were made subcutaneously over the abdomen as nearly as possible in the same region every time. The injection of 2 cc of 5 gdl distilled water solution of eosin G was repeated once every week as a rule and after 14 weeks, the dye solution was reduced to 1 cc. These injection procedures were, however, not strictly followed always but were somewhat modified depending on the condition of the animal. Eosin G solution was sterilized by heating before injection.

As a consequence of the injection the whole body of the animals showed a diffuse red vital staining which was noticeable one to two hours after the injection. In about twelve hours the maximum depth of color was reached, after which as the dye was excreted in the urine, the color of the animal gradually became paler, and at the end of forty-eight hours at the latest had completely disappeared.

In the course of the experiment 17 rats died early without showing tumor at the site of the injections, but one of them, dying on the 376th day, had a cysticercus sarcoma (Spindle cell sarcoma) of the liver, and two others, dying on 340th and 473rd day, fibroadenoma of the mammary gland.

Out of the remaining 3, which lived 503 days or more, 2 rats produced sarcoma at the site of the injections. Rat No. 20 in the table, dying on the 671st day with tumor at the site of the injections, also showed a mammary fibroadenoma. This, as well as other tumors found in rats dying earlier, may be regarded as of the spontaneous origin, not connected with the local sarcoma production. Pertinent data on these 2 rats may be seen from Table 5.

Table 5. Experiment V.

Rat No.	Sex	Exp. days	Eosin G		Body weight (end)	Tumor size (cm)	Histological diagnosis of tumor
			Total (g)	Inject. No.			
18	m	543	2.8	45	412	7.3×6.2×3.6	Spindle cell fibrosarcoma
19	m	648	3.65	61	185	No tumor	
20	f	671	3.65	61	170	3.3×3.2×1.7	Spindle cell fibrosarcoma

No. 18. A nodule measuring 4.0×2.0×1.5 cm was palpated on the 503rd day, which attain the size of 7.3×6.2×3.6 cm by the 543rd day, when the animal was killed in very weakened condition.

No. 20. On the 638th day a small finger-tip sized nodule was found which grew to the size of 3.3×3.2×1.7 cm by the 671st day. The animal died on that day.

These tumors were found in the subcutaneous tissue at the site of the injection. In general character they were much the same as rhodamine B sarcoma (Experi-

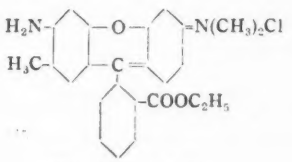
ment I), but tended to show more central necrosis. No macroscopic metastasis was found in any of the cases. No significant change was noted in internal organs at autopsy.

No change in the nature of liver surface, color or consistency was recognized in gross and showed histologically some hyperaemia and increase of Kupffer's stellate cells; no tumor or cirrhosis was observed. Spleen showed hyperaemia and sclerotic atrophy.

The both tumors were used for **transplantation experiments.** The transplantation from No. 18 took in 50% of the animals in the first generation, 25% in the second, 20% in the third, 40% in the fourth, and 30% in the fifth. Further transplantation was not made. Transplanted grafts usually produced palpable nodules in three or four weeks, and the rats died of tumors in one to two months on an average after transplantation. The transplantation from No. 20 did not succeed.

Failure of Sarcoma Production by Injections of Rhodamine 3 G, Fluorescein, Erythrosine or Violamine R.

Experiment VI. Rhodamine 3 G was tested in this experiment. Rhodamine 3G (Colour Index No. 753; $C_{25}H_{25}N_2O_3Cl$) is ethyl-ester of amino-methyl-di-methylamino-o-carboxy-phenyl-xanthenyl chloride, forming green crystaline powder, the watery solution of which has crimson-red color with a brown fluorescence.



(Rhodamine 3 G)

Experiment under the usual conditions was started with 20 normal rats of a mixed strain from the Saitama Prefecture, all weighing around 200 g.

The preparation of rhodamine 3 G used in the experiment was the product of the Bayer Co., Ltd., Germany.

Injections were made subcutaneously on the back of the rats at as nearly the same site as possible every time. The injection of 1 cc of 0.2 gdl distilled water solution of rhodamine 3G was repeated once every week as a rule and after about 7 months, the injection was reduced to two or three times monthly because of the slow absorption.

4 rats survived 300 days or more, one of the highest longevity surviving 367 days. (Table 6).

None of the animals developed tumor at the site of the injection.

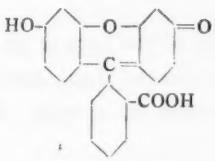
Table 6. Experiment VI.

Rat No.	Sex	Exp. days	Rhodamine 3 G		Final Body Weight (g)
			Total (mg)	Inject. No.	
5	f	348	64	32	230
6	f	367	66	33	255
7	m	367	66	33	242
8	m	367	66	33	227

The local tissue showed chiefly fibrinoplastic hypertrophy (5.3×4.0×1.5 cm) at the site of the injections. The cutis showed remarkable strong edema and atrophy of cells; mast cells appeared in a large number.

Spleen showed some hemosiderosis, karyorrhexis, atrophy, and fibrosis. Kidneys showed fibrosis and degeneration of interstitium to same extent. These were incidental findings, to which no special significance can be attributed.

Experiment VII. Experiment using fluorescein. Fluorescein is a mixture of dihydroxy-fluoran with hydroxy-o-carboxy-phenyl fluorone, forming orange-yellow powder.



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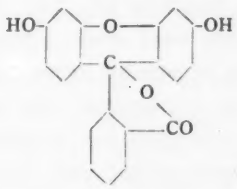


(Fluorescein)

Animals used were 15 rats of hybrid strain and 5 of Wistar strain weighing from 150 to 200 g.

The preparation of fluorescein (Colour Index No. 766; $C_{20}H_{12}O_5$) used in the experiment was the highest purity product (Merck), and olive oil was a product of the Iwaki Chemical Co..

Fluorescein is insoluble in water and therefore it was suspended in olive oil at the concentration of 5.0 percent, and was injected subcutaneously on the back of the rats in 1.0 cc amounts, delivered into as nearly the same site as possible. Because of the slow absorption injections were repeated once or twice monthly.

3 rat survived 200 days or more, one of the highest longevity surviving 564 days. (Table 7)

None of the animals developed sarcoma at the site of the injection.

In the course of the experiment, one of them, dying on the 88th day, had a fibroadenoma of the mammary gland (2.9×2.1×1.5 cm), and two others, dying on 175th and 564th day, adenoma of the hypophysis. Also one of them, dying on the 122nd day, had a cysticercus sarcoma (spindle cell sarcoma) of the liver. These

Table 7. Experiment VII.

Rat No.	Sex	Exp. days	Fluorescein		Olive Oil		Final Body Weight (g)
			Total (mg)	Inject. No.	Total (cc)	Inject. No.	
1	/	249	950	19	19	19	/
2	f	337	550	11	11	11	/
3	m	564	1050	21	21	21	212

tumors may be regarded as of the spontaneous origin, not connected with the experimental effect.

No significant change was noted in internal organs at autopsy. The local tissue showed chiefly an induration and fibrosis. The liver was generally atrophic.

Experiment VIII. Erythrosine (Colour Index No. 773: FD and C red No. 3; $C_{20}H_6O_5I_4Na_2+H_2O$) was tested in this experiment. The substance is said to be used in Japan for colouring foodstuffs officially permitted, for toilet-powder and also for colouring paper.

The preparation of erythrosine used in the experiment was the product of the Tokushu Chemical Co., Ltd., Tokyo.

Injections were made subcutaneously on the back of the rats at as nearly the same site as possible every time. The injection of 1 cc of 5 gdl distilled water solution of erythrosine was repeated once every week as a rule. Erythrosine solution was sterilized by heating before injection.

The experimental was begun with 20 albino rats of a mixed strain (Saitama strain), all weighing around 150 g.

7 rats survived 300 days or more, one of the highest logevity surviving 596 days. (Table 8)

None of the animals developed tumor at the site of the injection.

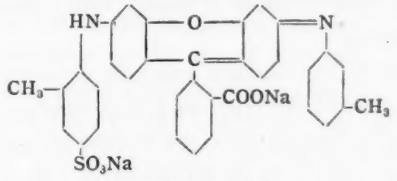
Table 8. Experiment VIII.

Rat No.	Sex	Exp. Days	Erythrosine		Final Body Weight (g)
			Total (mg)	Inject. No.	
11	f	314	1100	22	/
12	f	339	1150	23	/
13	f	367	1150	23	/
15	m	445	1650	33	/
18	m	459	1700	34	175
19	f	467	1700	34	/
20	f	596	2650	53	130

No significant change was noted in the local subcutaneous tissue as well as in

internal organs at autopsy. The liver was generally atrophic and histologically showed some hyperaemia. Spleen showed hyperaemia, sclerotic atrophy and fibrosis. Lymphnodes showed the proliferation of lymphocytes and histiocytic reticulum cells (containing dyes), and occasionally fibrosis and granulation formation were encountered.

Experiment IX. Lastly experiment was carried out using violamine R (Colour Index No. 758; $C_{34}H_{24}N_2O_6SNa_2$). Violamine R is a sodium salt of sulpho-di-o-tolyldiamino-o-carboxy-phenyl-xanthenyl, forming violet-red powder, the watery solution of which has violet-red color. The substance is said to be used largely for dying wool and silk; also for tinting and colouring paper.



(Violamine R)

The preparation of violamine R used in the experiment was the product of the Ciba Co., Ltd., Swiss.

Injections were made subcutaneously on the back of the rats at as nearly the same site as possible every time. The injection of 1 cc of 3 gdl distilled water solution of violamine R was repeated once every week as a rule and after about 4 months, the injection was reduced to once or twice monthly because of the slow absorption.

Experiment was started with 20 normal rats (Wistar strain descendants), all weighing around 130 g.

Table 9. Experiment IX.

Rat No	Sex	Exp. days	Violamine R		Final Body Weight (g)
			Total (mg)	Inject. No.	
8	f	300	960	32	124
10	f	346	960	32	144
11	m	350	960	32	/
13	m	394	990	33	/
14	f	418	1020	34	138
16	f	452	1050	35	/
17	f	460	1050	35	100
18	f	476	1080	36	126
19	f	477	1080	36	130
20	f	506	1140	38	140

10 rats survived 300 days or more, one of the highest longevity surviving 506 days. (Table 9) None of the animals developed tumor at the site of the injection.

However, in the course of the experiment, one of them, dying on the 346th day (f, Total violamine R: 960 mg, in 32 Injections), had a fibroma at the site of the injections (1.1×0.8×0.5 cm) and hypertrophy of thymus (2.0×1.5×1.5 cm), and five others, dying on 300th, 418th, 476th, 477th and 506th day, respectively, showed hypertrophy of thymus (small finger-tip or thumb-tip size). Also two others, dying on 394th and 452nd day, showed adenoma of the hypophysis (small pea size). With the exception of the local fibroma, all these may be regarded as spontaneous tumors.

The local tissue showed chiefly fibrotic changes with blue-violet color due to the absorption of the pigment.

No change in the nature of liver surface, color or consistency was recognized in gross and histologically only slight hyperaemia and tendency for cellular atrophy were noted. Spleen was generally atrophic and showed hemosiderosis and fibrosis. Lymphnodes showed the proliferation of lymphocytes and histiocytic reticulum cells; blood vessels were dilated.

Injections of Rhodamine B Mixed with Glucose and Light Green

The manner in which sarcoma develops at the site of rhodamine B injections are very much like the similar phenomena brought about by the injections of glucose (6) (7) (8) (9) (10) and other sugars (8) (10) (11) (12) (13) as well as of light green (14) (15). In view of this fact it may be of interest to investigate, what effect if any may be produced by adding such other substances as these in injecting rhodamine B.

Experiment X. In this experiment rhodamine B (R.B.) was injected together with glucose (G) and light green S.F. yellowish (L.G.) to the same site.

Animals used were hybrid Saitama strain of rat weighing from 150 to 300 g.

The sample of R. B. used was the same as in Experiment I and G. was the product of the Daiichi Chemical Co., Ltd., Tokyo, and L. G. that of the Merck. Injections were made subcutaneously over the abdomen as nearly as possible in the same region every time.

The injection of 2 cc of R.B. 5 mg, G. 400 mg and L.G. 10 mg in 2 cc of distilled water was repeated two to three times a week. The concentration of the solution was increased to R. B. 10 mg, G. 600 mg and L.G. 20 mg after 4 months. After about 13 months of repeated injections, when the rats showed injury at the site of injections on the back, injections were discontinued for a period of about 2 months, being resumed again after that period of interruption. The solution was

sterilized by heating before injection. Soon after completion of an injection the rats became vitally atained turning diffusely green-violet, and the staining became noticeable in one to two hours. In about twelve hours the maximum depth of color was reached, but as the dye was eliminated by kidneys, the color completely disappeared at the end of forty-eight hours. However, the subcutaneous tissues about the site of injection soon became deeply stained, and some green color persisted for many months after cessation of treatment. The kidneys, too, became deeply pigmented.

Of the 10 rats, with which the experiment was started, 9 rats survived the injection period of 289 days, receiving 81 injections during the period, 4 rats produced sarcoma at the site of the injections. (Table 10) There was another rat (No. 9) with small granuloma at the site of injections. Rat No. 10 in the table, killed on the 868th day, had a cysticercus sarcoma of the liver and fibroadenoma of the mammary gland, both of which may be regarded as of the spontaneous origin, not connected with the local sarcoma production.

The subcutaneous connective tissue became thickened and small hard consolidation of various sizes became palpable during the 8th to 15th months. These consolidations gradually turned into irregular nodules, which then, rapidly grew into large tumors The tumors were at first more or less diffuse, but as they increased in size, they became clearly demarkated from the surrounding connective tissue. The tumor, once developed, grew very rapidly to a very large size, and soon killed the animal.

Individual records of these 4 rats are as follows:

Table 10. Experiment X.

Rat No.	Sex	Exp. days	R.B. Total (mg)	G. Total (g)	L.G. Total (mg)	Inject. No.	Final Body Weight (g)	Tumor size (cm)	Histological diagnosis of tumor
1	f	289	600	42.0	1200	81	207	6.6×5.3×3.6	Rabdomyosarcoma
2	m	400	700	48.0	1400	91	172	No tumor	
3	m	498	750	51.0	1500	96	277	No tumor	
4	m	523	720	49.2	1440	93	361	5.3×3.7×2.6	Fibrosarcoma
5	m	536	790	53.4	1580	100	/	No tumor	
6	m	548	800	54.0	1600	101	142	1.9×1.8×1.2	Fibrosarcoma
7	f	565	800	54.0	1600	101	172	3.5×1.7×1.5	Spindle cell sarcoma
8	f	568	800	54.0	1600	101	/	No tumor	Granuloma
9	f	868	1080	65.4	1980	120	180	No tumor	

No. 1. On the 262nd day a hard nodule of the size of 2.8×2.5×1.4 cm was found, which grew to the size of 4.7×4.3×2.8 cm by the 274th day. The animal

died on the 289th day with the tumor measuring 6.6×5.3×3.6 cm.

No. 4. A small finger-tip sized nodule, first palpated on the 416th day, grew to the size of thumb-tip by the 492nd day. The tumor reached the size of 5.3×3.7×2.6 cm on the 523rd day, when the animal was killed in very weakened condition.

No. 6. On the 502nd day a hard nodule of the size of thumb-tip was palpated. The animal died on the 548th day with the tumor measuring 1.9×1.8×1.2 cm.

No. 7. A nodule measuring 3.0×1.7×1.5 cm was palpated on the 548th day. The animal died on the 565th day with the tumor measuring 3.5×1.7×1.5 cm.

These tumors were found in the subcutaneous tissue at the site of the injection. In general character they were much the same as rhodamine B sarcoma (Experiment I). No macroscopic metastasis was found in any of the cases. The transplantation was not made in any of the cases.

No significant change was noted in internal organs at autopsy. The liver showed generally anaemia and irregular congestion. Spleen showed anaemia, congestion and hemosiderosis. Kidneys showed round-cell infiltration. Lymphnodes showed hypertrophy of reticulum cells.

These tumors are diagnosed as fibrosarcoma, being composed chiefly of fibrinoplastic spindle cells. Only one tumor (No. 1) was classed as rabdomyosarcoma. Mitoses were numerous in most tumors, and in only a few were giant cells at all common, although nearly all tumors contained some.

Effect of Simultaneous Injections of Diatomaceous Earth on the Sarcoma Production by Rhodamine B

Since the time of Peyton Rous (1911) (17) it has been well established that local tissue derangement markedly favors the production of sarcoma in chickens by the filterable agent. Diatomaceous earth was used successfully for the purpose of inducing such a local tissue derangement. In the following experiment an attempt was made to see if a similar enhancement of sarcoma production can be brought about by simultaneous injections of diatomaceous earth.

Experiment XI. In this experiment rhodamine B was injected together with diatomaceous earth, using the hybrid Saitama strain of albino rats of 150 to 300 g body weight as usual.

The sample of diatomaceous earth (D. E.) used was the product of the Koso Chemical Co., Ltd., Tokyo.

The rats were given subcutaneous injections, at as nearly the same site as possible on the back, of 1 cc of watery solution of rhodamine B two to three times a week. The concentration of the solution was increased from 0.5 gdl to 0.75 gdl, and then to 1 gdl. 0.1g of diatomaceous earth was added to each 1 cc of the rhodamine B solution to be injected at the frequency of about once in a month

up to about 9 months in the course of the experiment.

The experiment was begun with 25 rats. 11 rats survived 300 days or more, one of the highest longevity surviving 659 days. (Table 11)

None of the animals developed tumor at the site of the injection.

Table 11. Experiment XI.

Rat No.	Sex	Exp. days	Rhodamine B		D. E.		Final Body Weight (g)
			Total (mg)	Inject. No.	Total (g)	Inject. No.	
1	/	305	590	73	0.8	12	247
2	m	316	607.5	68	0.75	11	280
3	f	364	660	80	0.8	12	177
4	f	369	745	88	0.9	12	162
5	m	408	710	85	0.8	12	170
6	/	419	795	93	0.9	12	224
7	f	463	647.5	82	0.75	11	132
8	f	510	855	99	0.9	12	178
9	m	579	895	103	0.9	12	190
10	m	586	840	98	0.8	12	275
11	m	659	920	106	0.8	12	155

The local tissue showed chiefly granuloma, ulcer and hyaline degeneration at the site of the injections. The liver showed generally hyperaemia. Spleen showed sclerotic atrophy, fibrosis and hemosiderosis. Lymphnodes showed hypertrophy, hemosiderosis and granulation. Kidneys showed round-cell infiltration.

It was first felt that simultaneous injections of diatomaceous earth, which is known to induce local granulation tissue, may facilitate and hasten the development of sarcoma. Contrary to this expectation, experimental results showed that diatomaceous earth not only failed to bring about the hastening of sarcoma production, but it might even inhibit the sarcoma producing process. Apparently, the formation of granulation tissue at the site of injection does not predispose the local tissue to sarcoma development.

Rhodamine B Feeding Experiment Using Rats

The purpose of this experimental was to ascertain whether rhodamine B feeding would cause pathological changes or even the development of malignant tumors when continued for a long period of time.

Experiment XII. Experiment was started with 21 normal rats (both sexes) of a mixed Saitama strain, all weighing around 150 g. They were maintained on rice diet, to which rhodamine B was mixed evenly. Usually, the amount of rhodamine B added was 0.13–0.2 per cent of the rice. The diet was supplemented with occasional dried fish, cod-liver oil, rape-seed oil, dried yeast and green vegetables.

The preparation of rhodamine B used in the experiment was the product of the Tokushu Chemical Co., Ltd., Tokyo.

The amount of rhodamine B added was 1.3 g per 1 kg of rice for about 7 months, increased to 2.0 g per 1 kg for the rest of the experimental period. The feeding of the dye was continued without interruption.

18 rats survived 300 days or more, one of the highest longevity surviving 661 days. (Table 12)

None of the animals developed tumor in any organ in the course of the experiment.

Table 12. Experiment XII.

Rat No.	Sex	Exp. days	Rhodamine B Total (g)	Final Body Weight (g)
1	f	307	6.07	/
2	m	334	6.7	/
3	m	335	6.73	/
4	m	342	6.94	/
5	f	374	7.87	159
6	m	379	8.02	/
7	f	383	8.14	163
8	f	399	8.62	/
9	m	443	9.91	/
10	f	443	9.91	132
11	f	451	10.15	141
12	f	496	11.41	/
13	f	510	11.80	154
14	m	551	12.91	/
15	f	554	12.97	146
16	f	565	13.3	170
17	m	612	14.5	151
18	m	661	15.7	138

No. 16 in the table showed hypertrophy of thymus (3.3×2.4×2.2 cm).

The gastric mucosa showed more or less hyperplasis, the liver generally showed slight degeneration of some parenchymal cells and some hyperaemia, and spleen showed hyperaemia and sclerotic atrophy, but none of these changes was sufficiently marked or constant to be regarded as significant.

Rhodamine B Injection Experiments using Mice

In experiments so far described, rats were exclusively used as experimental animals. Empirically, it was suspected that mice may be less suitable for the experiments of this sort, and this suspicion was verified experimentally.

Experiment XIII. The experiment was begun with 25 normal mice (both sexes) of a mixed Saitama strain, all weighing around 20 g. They were maintained on the usual laboratory diet of whole wheat with occasional supply of dried fish, cod-liver oil, rape-seed oil and green vegetables.

The preparation of rhodamine B used in the experiment was the product of the Kiriyama Co., Ltd., Tokyo. The mice were given subcutaneous injections, at as nearly the same site as possible on the back, of 0.1 cc of 0.5 gdl watery solution of rhodamine B two times a week. The concentration of the solution was reduced to 0.25 gdl after 1.5 months, and was increased to 0.5 gdl again after 4 months.

6 mice survived 150 days or more, one of the highest longevity surviving 260 days. (Table 13)

None of the animals developed tumor at the site of the injection.

Table 13. Experiment XIII.

Rat No.	Sex	Exp. days	Rhodamine B		Final Body Weight (g)
			Total (mg)	Inject. No.	
1	m	165	14.45	44	21.0
2	f	167	14.45	44	13.0
3	m	212	21.25	56	15.0
4	m	230	23.75	61	14.2
5	m	239	24.75	63	22.2
6	m	260	28.25	71	21.7

No significant change was found in the local tissue at the site of injections. Neither was any significant change noted in internal organs at autopsy. Only in rat No. 1 in the above table, there was a small granuloma in the liver, an isolated instance of no experimental implication.

Experiment XIV. The above experiment was repeated using 300 normal mice (Saitama strain), all weighing around 20 g, containing 120 males and 180 females. They were maintained on the same kind of the laboratory diet as before.

The sample of rhodamine B used was the same as in Experiment XIII. The mice were given subcutaneous injections, at as nearly the same site as possible on the back, of 0.1 cc of 0.5 gdl watery solution of rhodamine B two to three times a week. The concentration of the solution was increased to 0.7 gdl after 1 month, and was reduced to 0.5 gdl after another 1 month. After about 6 months of repeated injections, when the mice tended to became weak, the concentration of the solution was gradually decreased. During this stage of the experiment, injections were discontinued for a period of about 2 months, being resumed again after that period of interruption.

37 mice survived 200 days or more, of which 10 mice survived more than 280

days. One of the highest longevity surviving 363 days. (Table 14)

None of the animals developed tumor at the site of the injection.

Table 14. Experiment XIV.

Rat No.	Sex	Exp. days	Rhodamine B		Final Body Weight (g)
			Total (mg)	Inject. No.	
1	f	280	37.2	52	19.5
2	f	280	37.2	52	18.8
3	f	283	37.2	52	15.5
4	f	284	37.2	52	18.5
5	f	288	37.7	53	26.0
6	f	290	37.7	53	19.6
7	f	299	38.7	55	20.4
8	f	305	39.2	56	18.5
9	f	320	40.2	58	13.4
10	f	363	42.2	62	17.0

The local tissue showed no notable change. As to the internal organs, the liver was generally atrophic and histologically showed some hyperaemia, hemosiderosis and increase of Kupffer's stellate cells. Sometimes vacuolar degeneration of liver cells was noted, also amyloidosis, nuclear degeneration and karyorrhexis were noted in some cases. Kidneys showed round-cell infiltration and congestion. Spleen showed amyloidosis, hyperaemia, hemosiderosis and hypertrophy of follicle. These incidental histological changes cannot be regarded as of any special significance.

Rhodamine 6G Feeding Experiment using Mice

Supplementary to the main body of the experiments in which the dye stuff was repeatedly injected in rats, as already descrived in this paper, a prolonged feeding experiment was undertaken using mice. The dye used in this experiment was rhodamine 6G.

Experiment XV. Experiment was started with 40 normal mice (both sexes) of a mixed Saitama strain, all weighing around 20 g. They were maintained on rice diet, to which rhodamine 6G was mixed evenly. Usually, the amount of rhodamine 6G added was 0.01–0.05 per cent of the rice. The diet was supplemented with occasional dried fish, cod-liver oil, dried yeast and green vegetables.

The preparation of rhodamine 6G used in the experiment was the product of the Tokushu Chemical Co., Ltd., Tokyo.

The amount of rhodamine 6G added was 50 mg per 100 g of rice for about 6 weeks, reduced to 10 mg per 100 g for next 1 week, and increased to 20 mg per 100 g for the rest of the experimental period. The feeding of the dye was con-

tinued without interruption, mice being fed on the dye-diet *at libitum*.

12 mice survived 100 days or more, one of the highest longevity surviving 223 days (Total rhodamine 6 G : 90.6 mg).

None of the animals developed tumor in any organ in the course of the experiment.

The stomachs of the experimental mice showed more or less gastritis, hyperkeratosis, hyperplasia and papillomatosis. The liver generally showed degeneration of parenchymal cells. A marked degree of cirrhosis of the liver with nodular hyperplasis was found in one rat, dying on the 113rd day (Total rhodamine 6 G : 70.2 mg). Spleen showed hyperaemia, hemosiderosis and atrophy. Upon the whole, these pathological changes did not seem specially significant, but the case of liver cirrhosis with nodular hyperplasia might be worthy of note.

Discussion

Table 15.

Exp. No.	Name of Dye	Colour Index No.	Effective No. of Rats in Experiment	No. of Rats producing sarcoma
I	Rhodamine B	749	10	6
II	Rhodamine B	749	11	2
III	Rhodamine 6 G	752	7	4
IV	Fluorescein Sodium	766	8	2
V	Eosine yellowish	768	3	2
VI	Rhodamine 3 G	753	4	0
VII	Fluorescein	766	3	0
VIII	Erythrosine	773	7	0
IX	Violamine R	758	10	0

As may be seen from the above table, the tumors were produced by 4 of the 8 kinds of xanthene dyes tested. These dyes are all water soluble dyes except fluorescein. It is from 8 to 14 months after the beginning of the injections that tumor is first noticed as a hard nodule. The repeated injections of the dyes cause the subcutaneous tissue at the site to harden, and this gradually progresses to assume the form of a hard disk of tissue, which after one or two months grows into a tumor of from 5 to 6 cm in diameter, when it kills the rats. In practically all these cases tumors were produced only in a small percent of the rats used.

By the effective number in the above table is signified the number of rats surviving 245 days or longer in each experiment, the sarcoma usually appearing about this time if it is ever to appear.

The exact mechanism of this sarcoma producing process is not clear at present, but there is no doubt that the sarcoma is produced as a result of some stimuli

coming from the repeated injections of the dye solution. In this respect the present series of experiments belong to the same category with the previous investigation of Nishiyama (5), Takizawa (6), (12), (13), Arakawa (10), Schiller (14), Druckrey (18), Warabioka (19), Umeda (20), Watanabe (21), Tagashira (22), et al., in which subcutaneous sarcomas were obtained after subcutaneous injections of watery solution of glucose, glycogen, fluctose, galactose, lactose, light green S.F. yellowish, 4-dimethylamino-triphenylmethan, Janus green B, toluylene blue, formaldehyde, urotropin and monoiodoacetic acid. There is as yet no indication even to tell whether physical or chemical reactions play the deciding role, but it seems possible that carcinogenic action may have something to do with protein degeneration of local tissue. It may also be recalled that Oppenheimer (23), Turner (24), Druckrey (25), Zollinger (26), Laskin (27), et al. produced subcutaneous tumors in rats by subcutaneously implantation of the following polymer films: Cellophane, bakelite, dacron, polyethylene, polyvinyl chloride, pliofilm, nylon, polymethyl methacrylate, polystyrene, silk, etc. The mode of carcinogenesis in these cases is equally undeterminable.

In practically all these cases tumors were produced only in a small percent of the rats used. The consistently small percent of tumor production seems as though it is a universal rule for experiments of this type, and this apparent rule holds in the cases of xanthene dyes.

Attention may be called to the problems of coloring various food articles which have sprung up in the world lately. And in 1954 (28), by invitation of the "Deutsche Forschungsgemeinschaft", a meeting of scientists from West European countries took place in Bad Godesberg, with Butenandt as chairman. The purpose of the meeting was to stimulate international co-operation to prevent injury to health through toxic agents, particularly carcinogenic factors, in foodstuffs. The main emphasis was placed on food dyes. The participants were Butenandt, Druckrey, Warburg, Boyland, Euler, et al. In this connection the practical implication in human hygiene of the demonstration of carcinogenicity of certain xanthene dyes may perhaps be worth calling attention to, in view of the present wide use of some of these dyes as food dyes, and as coloring agent in various cosmetic preparations.

Summary

1. Sarcoma was induced by repeated subcutaneous injections of the following xanthene dye stuffs: Rhodamine B, rhodamine 6G, fluorescein sodium, and eosine yellowish.

2. Rhodamine 3 G, fluorescein, erythrosine, and violamine R did not show carcinogenic activity.

3. No notable change in internal organs was found in the rats under the conditions of these experiments.

4. The mechanism of the sarcoma producing process by the dyes is not clear at present.

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EXPLANATION OF FIGURES

Plate XV

Fig. 1. An example of sarcoma produced with rhodamine B.

Fig. 2. Sarcoma produced with rhodamine 6 G.

Fig. 3. Sarcoma produced with fluorescein sodium.

Fig. 4. Sarcoma produced with eosine yellowish.

Plate XVI

Fig. 5. A typical spindle cell sarcomatous picture of a tumor produced by the injections of rhodamine B.

Fig. 6. A polymorphous cell sarcomatous picture of a tumor produced by the injections of rhodamine B.

Fig. 7. Metastasis found in axillary lymph node (rat No. 16 in rhodamine B experiment).

Fig. 8. Histology of metastasis (Fig. 7.)

Plate XVII

Fig. 9. A typical spindle cell sarcomatous picture of a tumor produced by the injections of rhodamine 6 G.

Fig. 10. A polymorphous cell sarcomatous picture of a tomor produced by the injections of fluorescein sodium.

Fig 11. A spindle cell sarcomatous picture of a tumor produced by the injections of eosine yellowish.

Fig. 12. A tumor produced by the injections ot rhodamine B mixed with glucose and light green.

要　旨

Xanthene 色素による発癌実験

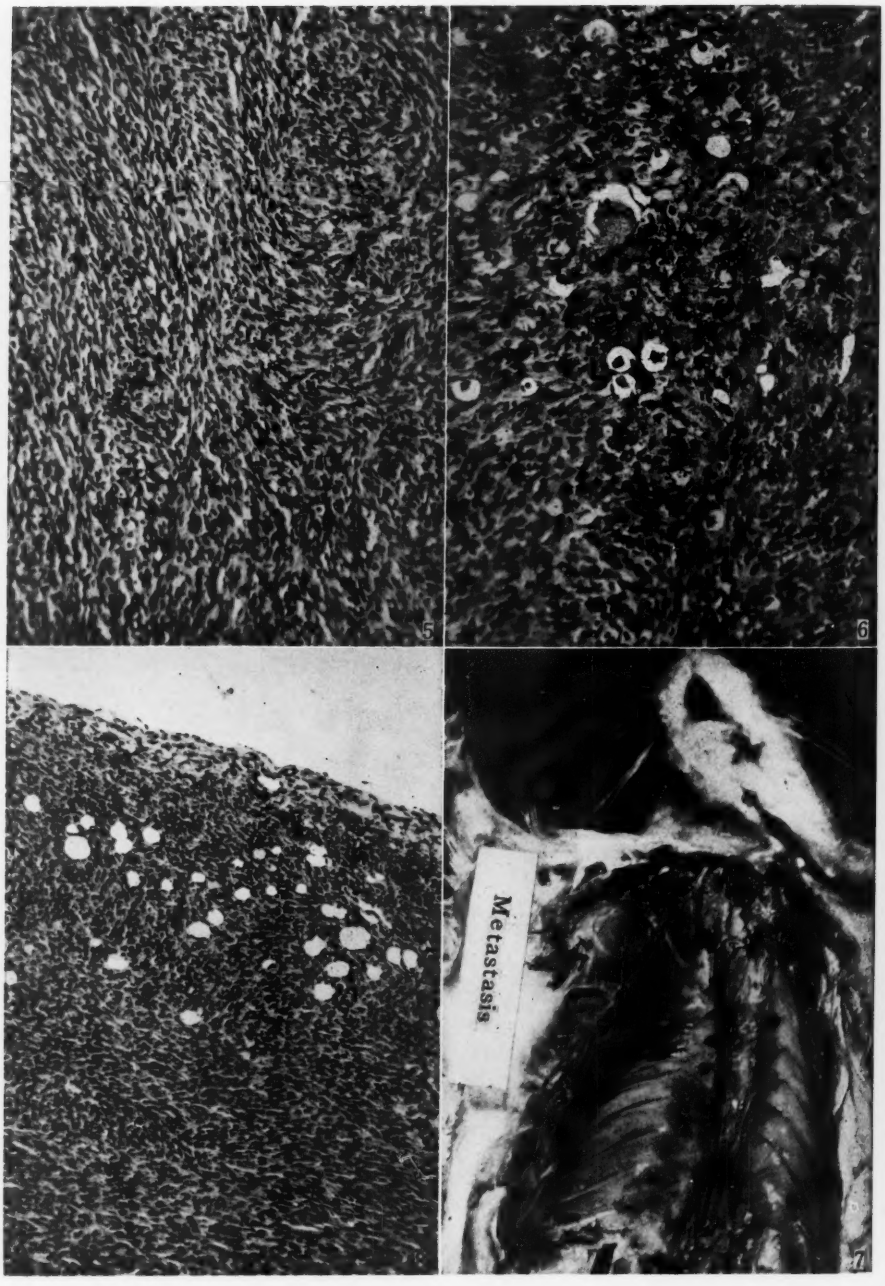
梅田真男
(癌研究所)

食用色素の問題に関しては現今癌の問題と関連して世界各国において，その重要性の声が高まって来た。現在日本において有毒色素の系列に属するローダミンBはしばしば飴，でんぶ，紅しようが，生菓子などの着色に用いられ問題の対称となっている。また化粧品の分野においてはローダミンB，エオシン，エリスロシンなどは，口紅，舞台用顔料などに用いられている。

今回の実験に用いた色素は，① Rhodamine B，② Rhodamine 6 G，③ Fluorescein Sodium，④ Eosine yellowish，⑤ Rhodamine 3G，⑥ Fluorescein，⑦ Erythrosine，⑧ Violamine R 等で，皮下反復注射の結果，ラッテの皮下に ①～④ の場合に，皮下肉腫を生成せしめた。

このように食用色素としてしばしば用いられ，また化粧品の色素として用いられている色素が発癌するという事実は，癌と色素との問題において将来何等かの意義があるのではないかと思われる。

R.B
1
R.6G
2
E.G
4
F.S
3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Metastasis
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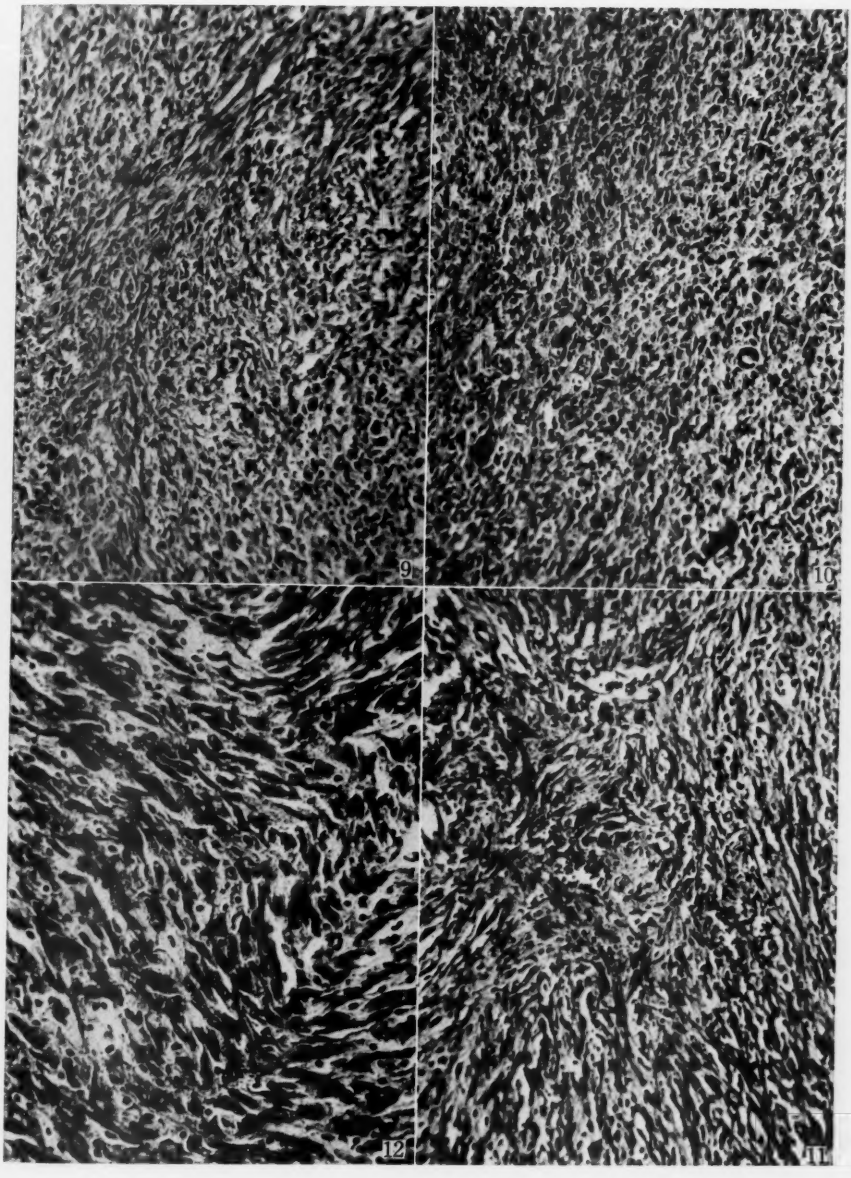
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[GANN, 47, 79~86; March, 1956]

REQUIREMENT OF COFACTOR FOR TRIPHENYLTETRAZOLIUM CHLORIDE REDUCTION BY SUCCINIC DEHYDROGENASE

TAKASHI SUGIMURA and TETSUO ONO
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The colorimetric method using TTC (triphenyltetrazolium chloride) for the determination of the succinic dehydrogenase activity in tissue was reported by Kun et al. (1), and applied by many other workers. We pointed out already that the succinic dehydrogenase activity in the animal tumor tissues measured by TTC reduction was far lower than in the normal tissues (2). The degree of decrease in the activity of tumor tissues was pronounced when it was measured by TTC reduction than when measured by the manometrical method of Schneider and Potter (3). So we presumed that some component was required for the TTC reduction in the presence of succinate as substrate, and that its concentration in tumor tissue should be low. On the other hand, Martin et al. reported that the TTC reducing activity in the presence of succinate as substrate was lost in the tuberculous guinea pig kidney homogenate, and the activity was restored by the addition of perchloric acid extract of normal guinea pig kidney to the homogenate (4). According to their later reports, they succeeded in isolating the desaminocoenzyme A as an effective substance for restoration (5, 6).

In our recent experiment using the pigeon breast muscle succinoxidase obtained by Keilin and Hartree's method, (10), we found that there is a certain unknown factor which is required for TTC reduction in the presence of succinate as substrate. Although we have not yet succeeded in the complete purification of the substance, its nature is different from desaminocoenzyme A in some points. In this paper we report on the results obtained in our experiments using pigeon breast muscle succinoxidase preparation.

EXPERIMENTAL

Materials used in this experiment were as follows:

Crude Coenzyme A (CoA) preparation was obtained from the hog liver by the method of Lipmann et al. (7) (in the first step of acetone precipitation, with specific acitivity at 5.4 units per mg). Glutathione was obtained from the laboratory of Kirin-Beer Co., Ltd, Tokyo. Diphosphopyridine nucleotide (DPN) of 90 per

cent purity and muscle adenylic acid (AMP-5) were purchased from the Nutritional Biochemicals Corporation, U. S. A. Thiamine pyrophosphate (TPP) and flavine mononucleotide (FMN) were purchased from the Light & Co., Ltd., England. Yeast adenylic acid (AMP-3) was a commercial sample from General Biochemicals, Inc. U. S. A. Flavine adenine dinucleotide (FAD) of 90 per cent purity was obtained through the courtesy of Prof. Y. Shimazono, Tokyo University. Adenosine triphosphate (ATP, potassium salt) was prepared according to Le Page's method (8). Cytochrome c was prepared from the horse heart by Keilin and Hartree's method (9). Denaturated globin was prepared by the method of Keilin and Hartree (10).

Kun's original method was used for the determination of succinic dehydrogenase by means of TTC. The triphenyl formazan, which was formed in the reaction which proceeded aerobically for 30 minutes at 37°C without shaking, was extracted with acetone or, occasionally, ethyl acetate.

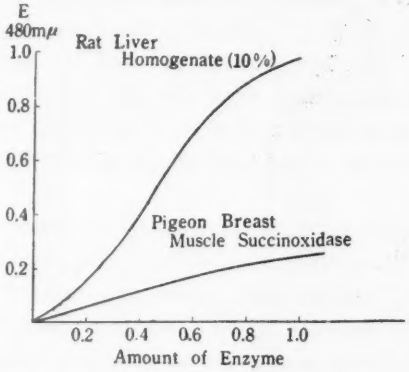
Pigeon breast muscle succinoxidase preparation was obtained by the method of Keilin and Hartree with slight modification. The enzyme could be used for a few days when kept in a refrigerator, but this was diluted before using to the suitable concentration with 1/100 M phosphate buffer, pH 7.4. Boiled extract of tissue was prepared as follows: Water was added to the tissue at the ratio of one to one, and homogenized. Homogenate was boiled for 15 minutes, after which the supernatant was separated by centrifugation at 6000 rpm.

Crude CoA preparation was fractionated with the Dowex 1, anion exchange resin, cross-linkage 2,200-400 mesh, which was obtained from the Dow Chemical Company, U. S. A. Resin was washed alternately three times with 10 times the volume of N HCl, water and N NaOH, and finally twice with 10 times the volume of N HCl, and with 50 times the volume of water. The column length of resin was 16.5 cm, and the velocity of elution was 0.25 ml per minute. As elution solution N/200 HCl, N/100 HCl and N/50 HCl were used successively.

Phosphorus, ribose and sulfur were determined colorimetrically by Allen's method (11), Meijbaum's method (12) and Klein's method (13), respectively. SH was determined by amperometric titration method as described by Goldzieher et al. (14) Pantothenic acid was assayed microbiologically by using double enzymes method of Novelli et al. (15). *Lactobacillus arabinosus* was kindly supplied by courtesy of the Applied Microbiological Institute of Tokyo University. In part of these experiments, the manometrical assay of succinoxidase of Schneider and Potter (3) was applied.

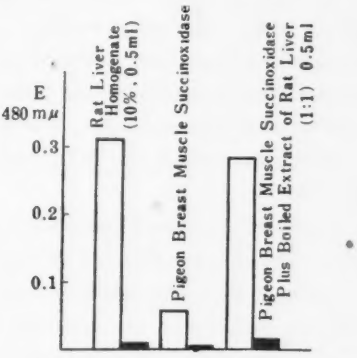
RESULTS

Fig. 1. The Comparison of TTC Reducing Activity between Rat Liver Homogenate and Pigeon Breast Muscle Succinoxidase.



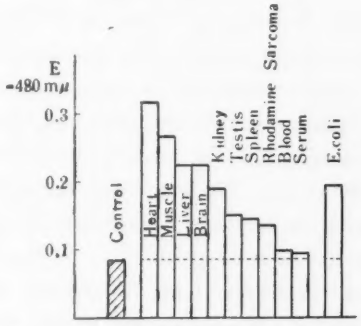
Assay system: Total volume 3.0 ml, 1/10M phosphate buffer, pH 7.4, 1.0 ml, 1/5 M sodium succinate 0.5 ml, 0.2% TTC 0.5ml. 37°C, 30 minutes.

Fig. 2. The Response of Addition of Boiled Extract to Pigeon Breast Muscle Succinoxidase



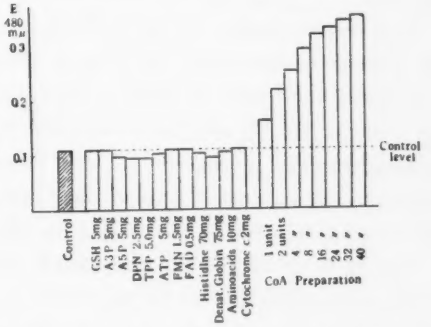
Experimental conditions were the same as described in Figure 1. Black column represents no addition of succinate. Both enzymes have the same activity manometrically.

Fig. 3. The Response of Addition of Various Tissue Boiled Extract to Pigeon Breast Muscle Succinoxidase



Experimental conditions were the same as described in Figure 1. 0.5 ml of boiled extract was added.

Fig. 4. The Response of Addition of Various Coenzymes and Other Substances to Pigeon Breast Muscle Succinoxidase.



Experimental conditions were the same as described in Figure 1.

The comparison of TTC reduction activity between the rat liver homogenate and the pigeon breast muscle succinoxidase is illustrated in Figure 1. The amount of enzyme solution which have the manometirically same activity is plotted on abscissa. 1.0 on abscissa represents 1.0 ml of 10 per cent rat liver homogenate. It is clear that the pigeon breast muscle succinoxidase hardly reduces TTC in the presence of succinate. In Figure 2 is represented the response which takes place when the boiled extract of normal rat liver is added to the pigeon breast muscle succinoxidase preparation. The rat liver homogenate and the pigeon breast muscle succinoxidase used in this experiment have the same activity if measured manometrically. The addition of boiled extract of rat liver to the latter restores completely the TTC reducing activity to the level of the rat liver homogenate.

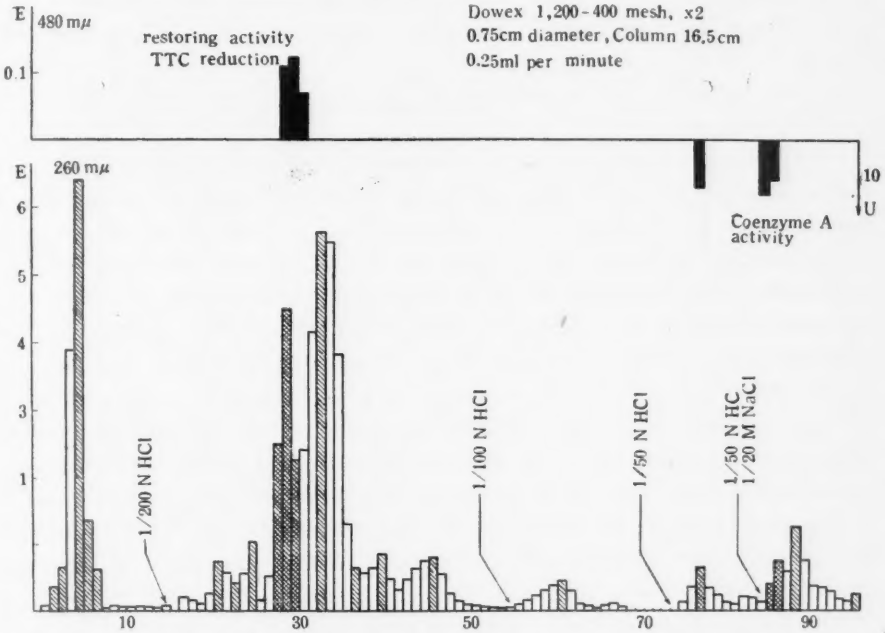
Next, we examined the distribution of this substance in the various organs of rat. The results are given in Figure 3. The concentration of this active substance is high in the order named: heart, brain, kidney, testis, spleen and tumor (Rhodamine sarcoma of rat, fibrosarcoma). In blood and serum it is hardly recognized.

In Figure 4 is illustrated the response which takes place when various coenzymes or other substances are added in order to restore the TTC reducing activity of the pigeon breast muscle succinoxidase. It is sure that glutathione, AMP-3, AMP-5, ATP, FMN, FAD, TPP and DPN are all inactive. Although Keilin and Hartree, who assayed manometrically, reported histidine and denaturated globin as the activator of the pigeon breast muscle succinoxidase, these substances are also inactive. Cytochrome c and amino acids mixture (composed similarly to human serum protein) are likewise inactive. But crude CoA preparation is very active in the restoration and the activity increases with the amount of CoA preparation.

The results of fractionation of crude CoA with Dowex 1 is presented in Figure 5. Ordinate shows its extinction at 260 mμ and abscissa shows the fraction tube number. Each tube contains 5 ml of eluent. Active substance is contained only in No. 28, No. 29 and No. 30 of these tubes, which compose one prominent peak with ultraviolet absorption. These three tubes have not the CoA activity measured by the method of Kaplan and Lipmann (16) at all. The coenzyme A activity is recognized in No. 77, No. 85 and No. 86, in which the recovery is considerably low.

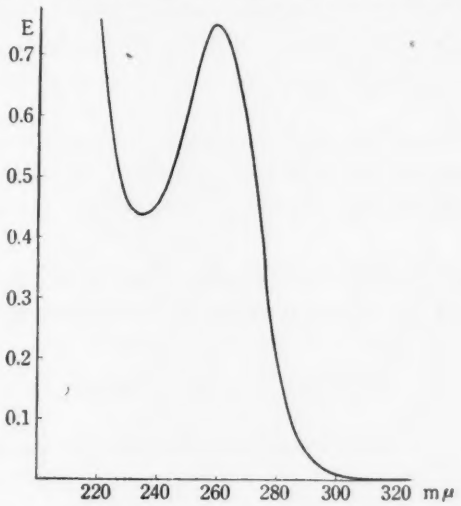
Figure 6 represents the absorption curve of No. 29 tube at pH 2.5. It has the maximum extinction at 257 mμ, which is shifted to 260 mμ at pH 7.0 and pH 10.0. It has the minimum extinction at 234 mμ at pH 2.5. We recognized in this fraction, colorimetrically, ribose, phosphorus and sulfur. SH group was recognized amperometrically in oxidized state which is reduced by Na_2SO_3 with alcohol. But, contrary to our expectations, pantothenic acid is not recognized in the bioassay

Figure 5. Fractionation of Crude CoA Preparation with Dowex 1 Resin



Shaded bars (tubes) in this figure were determined about the restoring activity. Upper figure represents the restoring activity in TTC reducing and CoA activity.

Figure 6. Absorption Curve of Tube No. 29 at pH 2.5



using *Lactobacillus arabinosus*. On the other hand, in the fractions which have CoA activity, pantothenic acid is contained in the amount calculated as pure CoA.

Discussion

From the results of the above experiments, it is clear that a certain unknown factor is required for TTC reduction in the presence of succinate by the pigeon breast muscle succinoxidase. This factor is contained in boiled extract of tissues and crude CoA preparation, and is probably not an ordinary coenzyme such as DPN and others. Although Martin et al. reported desaminocoenzyme A as the activator of tuberculous gulnea pig kidney for TTC reduction, the factor in our experiments is different in that it does not contain pantothenate. And although we recognized SH group in this active fraction, the possibility of contamination of next fractions which contain more SH group and have no activity must be considered. Furthermore, the absorption curve of ultraviolet region of this fraction does not agree with the absorption of hypoxanthine which is contained in desaminocoenzyme A. We must point out that the concentration of this substance is highest in heart, although the concentration of CoA is highest in liver and relatively low in heart. Recently, Bril et al. reported that DPN increased the TTC reduction with rat liver particles in the presence of succinate (17). Although we could confirm their results, this phenomenon was not found in the case of pigeon breast muscle succinoxidase preparation. And in our active fractions, we did not find the absorption at 340 mμ when reacted with CN, which is characteristic of the N-substituted nicotinamide compounds (18). It is sure that active substance in our experiments is neither DPN nor TPN, and at the present time we think we can rule out the DPN as the activator in the case of pigeon breast muscle succinoxidase.

In practice, the assay method of succinic dehydrogenase using TTC must not be applied to compare the activities among different organs or among different cellular fractions, because the concentration of this cofactor may become a rate limiting element.

We are now working towards the isolation of this substance in pure state. We are very interested in the biological meaning of this substance.

Summary and Conclusion

1. The pigeon breast muscle succinoxidase prepared by the method of Keilin and Hartree requires the cofactor for the reduction of TTC in the presence of succinate.

2. This factor is contained in the boiled extract of various tissues. The concentration of this factor in tumor tissue is very low.

3. This factor is also contained in high concentration in crude CoA preparation obtained from hog liver.

4. From crude CoA preparation, one peak active for TTC reduction was obtained with fractionation using Dowex 1 resin. It contains ribose, phosphorus, sulfur and base having the maximum absorption at 257 mμ at pH 2.5, but it has not pantothenate.

5. This factor in our experiment is different from DPN or desaminocoenzyme A.

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要　旨

コハク酸脱水素酵素による TTC の還元に対しての助酵素の必要性

杉村　隆，小野哲生（癌研究所）

癌組織では TTC の還元でコハク酸脱水素酵素を測定すると，正常に比して非常に値が低くでることから出発し，鳩胸筋から調製したコハク酸酸化酵素を用いることにより，コハク酸脱水素酵素と TTC の間に介在する未知の助酵素を確認した。これは DPN 等既知のものでもなく，また Martin 等によって結核症のモルモット腎でコハク酸脱水素酵素と TTC の間に介在するとのべられた Desaminocoenzyme A とも異なるものである。

（本研究は文部省科学研究費による。）

[Gann, 47, 87~90; March, 1956]

ERYTHROCYTE PORPHYRIN IN BLOOD OF TUMOR BEARING ANIMALS

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(Cancer Institute, Japanese Foundation for Cancer Research, Tokyo)

It is well established that less cytochromes and catalase are contained in tumor cells that in normal cells. And also, in the tumor-bearing animals, the depression of the liver catalase activity and anemia are the most universal and remarkable phenomena (1). Fukuoka and Nakahara suggested that the depression of liver catalase activity after injection of toxohormone into mouse may be related to the disturbed iron metabolism (2).

In view of these facts, since heme, which is the prosthetic group common to hemoglobin, catalase and cytochrome b, is the iron porphyrin, composed of protoporphyrin, it is very important to clarify the porphyrin metabolism in tumor cells and the tumor bearing animals. In this preliminary report is described the fact that free porphyrin of the erythrocyte (EP) is increased in the tumor bearing animals which have anemia.

Material and Methods

Male white rats, Saitama mixed strain, were used in all the experiments. Tumors used were rhodamine sarcoma (fibrosarcoma) (3) and m-toluylenediamine induced sarcoma (fibrosarcoma) (4), both transplanted subcutaneously. Animals were killed by causing them to bleed from the axillar arteria 14-27 days after transplantation when tumor weight reached 20-50 g.

Hemoglobin concentration was determined colorimetrically after mixing 0.5% sodium carbonate(5), and hematocrit values were determined in the usual manner. The free porphyrin of erythrocytes was determined by the method which is a slight modification of that of Schwartz and Wikoff(6). Finally, porphyrin extraced in HCl solution was determined at 411 mμ by Beckman spectrophotometer. In this preliminary experiment, copro- and protoporphyrin were not determined separately. Recrystallized protoporphyrin prepared by the method of Grinstein and Watson (7) was used as the standard sample.

Results

Full data are tabulated in Table 1. Figure 1 represents the mean values of hemoglobin content, hematocrit value and erythrocyte prophyrin. In tumor-bearing

animals, the decrease of hemoglobin content and hematocrit value are remarkable, while, in contrast to this, erythrocyte porphyrin level is much increased. If the ratio of EP to hemoglobin content or to hematocrit value in two groups of animals, control and tumor-bearing, is compared, the difference between them becomes

Table 1. Hemoglobin content, hematocrit value and free erythrocytic porphyrin in blood of normal and tumor-bearing animals.

	No.	Name of tumor	Body weight, gm	Tumor weight, gm	Days after transplantation	Hemoglobin (Hb) gm/dl	Hematocrit (Ht) %	Erythrocyte porphyrin (EP) μg/dl	EP/Ht	EP/Hb	Hb/Ht
Control animals	1	—	162	—	—	12.0	39.8	54.5	1.36	4.54	3.32
	2	—	99	—	—	11.8	40.7	56.0	1.36	4.75	3.45
	3	—	93	—	—	13.0	44.3	44.0	0.98	3.46	3.41
	4	—	90	—	—	13.7	45.9	70.5	1.52	6.70	3.35
	5	—	125	—	—	12.6	42.3	55.0	1.28	4.48	3.44
	6	—	110	—	—	13.1	41.9	34.8	0.83	2.67	3.20
	7	—	97	—	—	11.5	42.8	32.0	0.74	3.78	3.72
	8	—	80	—	—	11.7	39.6	37.0	0.93	3.17	3.38
	9	—	160	—	—	13.2	45.0	31.4	0.76	2.38	3.41
	10	—	150	—	—	12.4	43.3	32.5	0.75	2.62	3.49
	11	—	100	—	—	14.9	51.7	39.0	0.75	2.64	3.46
	12	—	97	—	—	14.2	48.9	29.2	0.59	2.07	3.44
Mean						12.8	43.8	42.9	0.90	3.60	3.42
Tumor-bearing animals	13	Rh	81	54	24	10.9	41.1	164.0	3.96	15.10	3.76
	14	Rh	62	25	24	13.3	51.4	79.0	1.53	5.98	3.87
	15	Rh	100	22	17	9.5	36.0	53.5	1.48	5.75	3.79
	16	Rh	109	28	17	11.3	37.8	74.5	1.90	6.41	3.34
	17	Rh	93	41	23	12.9	46.0	69.5	1.50	5.42	3.57
	18	Rh	78	43	23	11.3	39.2	46.5	1.17	4.13	3.47
	19	MTD	85	39	27	10.5	40.2	114.0	2.82	10.90	3.83
	20	MTD	105	15	27	13.7	42.5	74.5	1.68	5.36	3.10
	21	MTD	104	51	22	12.4	34.7	78.5	2.28	6.35	2.80
	22	Rh	80	32	14	8.2	43.3	87.0	2.00	10.70	5.28
	23	Rh	95	27	14	6.0	34.5	55.0	1.58	9.15	5.75
Mean						10.9	40.6	81.5	1.99	7.75	3.87

(Abbreviations: Rh, Rhodamine sarcoma: MTD, m-Toluylenediamine induced sarcoma)

further pronouced. The means of these ratios are given in Figure 2.

Figure 1. Average Value of Hemoglobin Content, Hematocrit Value and Free Erythrocyte Porphyrin in Blood of Normal □ and Tumor-bearing Animals ▨.

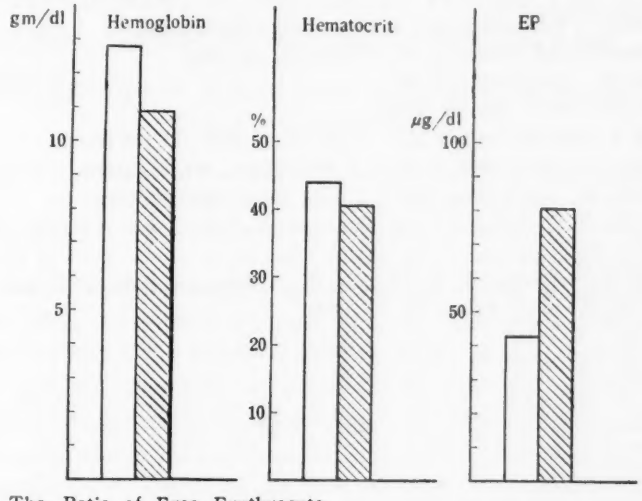
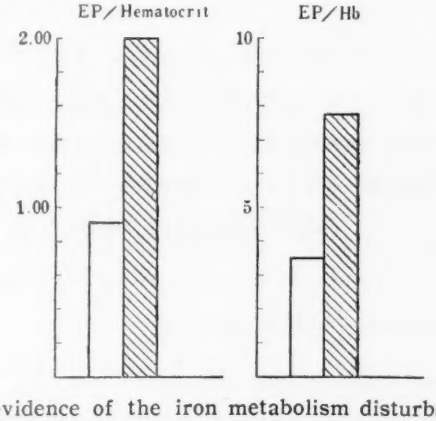


Figure 2. The Ratio of Free Erythrocyte Porphyrin to Hemoglobin Content or Hematocrit Value in Blood of Normal □ and Tumor-bearing Animals ▨



Summary and Discussion

From the results described above, it is clear that the level of EP, most of which is protoporphyrin, rises in the blood of tumor bearing animals. Protoporphyrin combines with iron, and changes to protoheme, which is the prosthetic group of hemoglobin and catalase. Although, in the case of tumor bearing animals, the possibility of supressed synthesis of protein moiety of hemoglobin cannot be neglected, accumulation of protoporphyrin observed here may be considered as an evidence of the iron metabolism disturbance. It is known that in iron deficient anemia, EP level is very high when compared with that in the pernicious anemia (8). Furthermore, it was reported that lead poisoning affected the mechanism of the iron incorporation into protoporphyrin, and increased the FP (9). Recently, Schwartz et al. reported of the presence of erythrocytic and hepatic types in

porphyria (10). Also in the tumor bearing animals, high level of protoporphyrin in liver was recognized in onr laboratory. This new finding will be reported in our subsequent paper.

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要　旨

担癌動物血液のポルフィリン

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ローダミン肉腫および m-トルイレンヂアミン肉腫を移植したラットの血液は，貧血を示すとともに，血液赤血球の遊離ポルフィリンの増加を認めた。増加の程度は大体正常動物の2倍である。この新事実は癌組織でチトクローム，カタラーゼ等が少いこと，担癌動物で貧血，肝カタラーゼ減少が見られること等と関連し，担癌動物のポルフィリン代謝障害を思わしめるもので興味深い。

(本研究は文部省科学研究費による)

[GANN, 47, 91~95; March, 1956]

THE DETERMINATION OF D-GLUTAMIC AND D-ASPARTIC ACIDS CONTENT OF MALIGNANT TUMORS AND NORMAL TISSUES BY MEANS OF NEW OXIDASE

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The reports by Kögl and co-workers[1] on the characteristic occurrence of amino acids of unnatural configuration, especially D-glutamic acid, in malignant tumors in man and rabbit have been discussed by several authors, and the majority of them[2] failed to confirm the result obtained by Kögl. However, strictly speaking, the isolation procedures used by these authors are not adequate to prove that their results are really contradictory to Kögl's conclusion. Lipmann and co-workers[3] used D-amino acid oxidase preparation of Krebs for the determination of the D-amino acid content of cancer tissues with the result quite different from that of Kögl. However, this enzyme preparation oxidizes almost all the D-amino acids more rapidly, and has much weaker activity towards D-glutamic acid, so that it is impossible to estimate D-glutamic acid content of cancer tissues. Afterwards some papers using the isotopic technique for the purpose have appeared.[4] So far as we know, the direct determination of D-glutamic acid content of tumours has not ever been reported.

In looking for the microorganisms for the purpose of studying the metabolism of D-amino acid, we found that the fungus belonging to **Aspergillus ustus** metabolizes D-glutamic acid considerably.* When the fungus was grown in the medium containing D-glutamic acid as a sole source of nitrogen, its dried cell or the crude extract oxidized D-glutamic and D-aspartic acids specifically and quantitatively according to the following equation:

$$\text{D-glutamic acid} + \frac{1}{2}O_2 \longrightarrow \alpha\text{-ketoglutaric acid} + NH_3$$

$$\text{D-aspartic acid} + \frac{1}{2}O_2 \longrightarrow \text{oxalacetic acid} + NH_3$$

We have, therefore, used this enzyme preparation for the determination of the D-glutamic and D-aspartic acids content of the hydrolysate of cancer and normal tissues.

* The details of this work will appear in Bulletin of the Agr. Chem. Soc. of Japan

Materials and Methods

Materials: D-, L-glutamic acid, DL-aspartic acid were kindly supplied by Ajinomoto Co., Ltd. Cancer tissues, both human and animal, were kindly supplied by The Cancer Institute, the Japanese Foundation for Cancer Recearch.

Preparation of amino acid solutions from normal and cancer tissues: Amino acid solutions were prepared according to the procedurses described by Kögl. Tissue was prepared in Waring blender in 0.6% sodium chloride solution of the volume six times as large as the tissue, and stored at 0°C for 24 hours. After centrifugation, the salt solution was mixed with four volumes of ethanol. The resulting precipitate was washed with 80% ethanol, and was heated at 110°C to dryness. Then it was hydrolyses for seven hours in the 20% hydrochloric acid of the weight twenty times as large as the tissue. From the hydrolysate, the excess HCl was expelled as far as possible by concentrating to dryness several times under reduced pressure. The sample was neutralized in aquous solution and diluted, and was used in the experiment.

Enzyme preparation: The mycelia of **Aspergillus ustus** strain f incubated in the medium (crude glucose 2%, D-glutamic acid 0.5%, K_2HPO_4, 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, $FeSO_4$ trace) for 4–5 days on a shaker were employed. The mycelium was removed from the medium by filtration through gauze, washed with distilled water, and pressed by hand to remove the excess water through cotton cloth. The pressed mycelium was stored at −20°C for 3–24 hours and dried over $CaCl_2$ in vacuum. 15 g of the dried mycelium was ground in a motor and was extracted with 150 ml of borate-phosphat buffer pH 8.0 for 30 minutes. The resulting material was filtered through cotton cloth, the residue was again extracted with 100 ml of the buffer and was filtered. Thus, 200 ml of the crude extract was gained. 30 ml of 1% protamine solution (pH 6.0) was added to 200 ml of the crude extract to exclude nucleic acid, and then solid ammonium sulfate was added to the supernatant little by little to 60% saturation. The precipitate was centrifuged at 10,000 r.p.m. for 30 minutes and dissolved in 30 ml of the borate-phosphate buffer.

This solution was used as the enzyme preparation. This enzyme preparation oxidizes strongly D-glutamic and D-aspartic acids, but does not effect at all any of other DL-amino acids, α-ketoglutaric acid and oxalacetic acid.

This enzyme preparation yields 1 mole of ammonia and α-ketoglutaric acid quantitatively from 1 mole of D-glutamic acid and $\frac{1}{2}$ mole of oxygen.

Assay method of D-glutamic and D-aspartic acids: The amount of D-glutamic and D-aspartic acids were determined by measuring the oxygen up-take resulting from the oxidation of these D-amino acids by the use of Warbing respirometer

at 30°C. Each experimental vessel contained 0.2–1.0 ml of a sample with 1.0–2.0 ml of borate-phosphate buffer (pH 8.0) in the main compartment, 0.2–0.4 ml of the enzyme preparation in the side arm, and 0.2 ml of 20% KOH solution in the center-well with 1 cm^2 of filter-paper.

All experiments were carried out in parallel with control experiments and recovery tests with 1–2 μM of D-glutamic acid. Readings were taken as usual for 1–2 hours until the pressure changes in all of the control, experimental, and recovery test flasks became equal to each other. The values of the oxygen up-take have been corrected for any oxygen consumption by the control experiment. The value obtained by this manometrical method shows the total ammount of D-glutamic and D-aspartic acids. In order to determine each D-amino acid separately, fractional determination of α-ketoglutaric acid and oxalacetic acid in the reaction mixture must be carried out. In our case, however, as the total amount of D-glutamic and D-aspartic acids was very small, the frctional determination of these organic acids was not carried out.

Assay method of L-glutamic acid: L-glutamic acid was determined by the manometric method, using L-glutamic acid decarboxylase from Escherichia coli-crooks.[5]

Results and Discussion

Determination of L-glutamic acid and the total amount of D-glutamic and D-aspartic acids were carried out for three kinds of normal tissue and nine kinds of cancer tissue.

The results are shown in Table 1.

The values in Columm 5 show the ratio in percentage of the total amount of D-glutamic and D-aspartic acids. Therefore, the ratio of D-glutamic acid to D-, L-glutamic acid must be smaller than these values. Except for transplantable ascites hepatoma, all values of Columm 5 are less than 4 %. Since it has been widely accepted that 3–6% of L-glutamic acid is racemized by hydrolysation with HCl, the presence of D-glutamic acid by 4 % does not show the occurrence of D-glutamic acid in the original tissue. As to transplantable ascites hepatoma, though the value in Columm 5 does not seem to be very small, still this value is not so large as that reported by Kögl. The small and uniform values of D-glutamic acid observed in the variety of hydrolysates examined for normal and cancer tissues are not characterized by the presence of D-glutamic acid.

Summary

A new enzymatic method of determination of D-glutamic acid has been applied to the determination of D-glutamic acid content of cancer tissues.

Table 1. D-Glutamic and D-aspartic acids content of tumors and normal tissues.

Columm 1	2	3	4	5	6
Material	Crude protein	L-glutamic acid	D-glutamic acid +D-aspartic acid	columm 4×100/ columm 3+4 ×100	Recovery of D-glutamic acid
	mg	mg	mg	%	%
Normal tissues:					
Muscle (horse)	1000	59.5, 57.9	1.3, 1.4	2.2±0.1	104
〃 (rat)	670	77.1, 73.8	1.7, 1.4	2.0±0.2	71
Liver (mouse)	230	27.0, 26.9	0.4	1.5	92
Tumor tissues:					
N-F sarcoma (mouse)	270	27.5, 27.1	0.6, 0.8	2.5±0.5	99
Rhodamine sarcoma (rat)	490	42.3, 44.3	1.4	3.1	99
Same, necrotic part	100	13.3	0.4	2.9	97
Ehrlich ascites carcinoma (mouse)	580	60.1, 57.4	0.5, 0.4	0.7±0	108
Ehrlich solid carcinoma	430	44.7, 46.8	1.8, 1.7	3.7±0.2	107
Ehrlich ascites fluid	840	101.2, 90.2	1.6, 2.2	1.9±0.4	90
Ascitic hepatoma (rat)	300	36.6, 33.6	2.6, 2.7	6.9±0.5	95
Primary liver.carcinoma (man)	1600	96.0, 96.0	2.6	2.7	105
Kidney adenocarcinoma (man)	1000	70.6, 64.2	2.0	2.9	78

It has been shown that cancer tissues are not characterized by the presence of D-glutamic acid.

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要　旨

新酸化酵素による悪性腫瘍及び正常組織の D-グルタミン酸及び D-アスパラギン酸の定量

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最近，著者等は新に土壌より分離したアスペルギルス　ウストスに属する一菌株より，D-グルタミン酸及び D-アスパラギン酸を酸化するが，他の DL-アミノ酸を酸化しない酵素の粗抽出液を得た。この酵素標品は D-グルタミン酸及び D-アスパラギン酸を定量的に酸化するので，これらのアミノ酸の定量に用いられる。著者等はこの方法により長い間問題となっていた癌細胞中の D-グルタミン酸の存在について調べたが，第一表に示す通り，D-グルタミン酸は癌細胞中に特に多く存在するものではなかった。

[GANN, 47, 97~103; March, 1956]

INHIBITION OF EXPERIMENTAL PRODUCTION OF LIVER CANCER BY TOBACCO TAR

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Recently the increasing frequency of primary cancer of the lung has aroused a great interest on the basis of a clinical and statistical investigations and many attempts have been made with tobacco products to induce cancers in experimental animals. Some workers attempted to induce pulmonary tumors in animals with tobacco smoke (1-5), and others applied a condensate of tobacco smoke to the skin of animals (6-10). And some of the investigators succeeded in inducing pulmonary tumors in mice with tobacco smoke and in proving condensed cigarette smoke as a carcinogen for mouse epidermis. Assuming that some of the tobacco tar may be dissolved into saliva and may be swallowed during smoking, this investigation was made to test the effect of tobacco tar feeding on the experimantal production of liver cancer by azo-dye feeding. The details of the experiment are given in the following pages.

Experimental Results

Preparation of tobacco tar: A mixture of Japanese popular brand of domestic cigarettes was placed in an iron retort and heated for 4-6 hours with a gas burner. The temperature during the combustion of the cigarettes was about 800°C. The distillate consisted of a yellowish brown liquid and of a very dark brown oily liquid almost similar to that described by the previous investigators (7, 9). As we assumed that the effect of tobacco smoke might be owing to summation effect of tar or alkaloid fraction the whole condensate was used. Then, the whole distillate was diluted with the same amount of polyethylene glycol (400) and was used as dietary supplement.

Experiments 1 and 2: Two groups of male albino rats of Wistar strain were used in the experiment. In the first group (tobacco tar fed), rats were maintained on the rice diet, containing of 2.5 per cent of whole tobacco tar dissolved in polyethylene glycol, 2 per cent of fish powder, 0.5 per cent of sodium chloride and a small amount of cod liver oil. Green vegetables were given thrice a week, and water was allowed in unlimited amount. In the second group (control), rats were fed on the same rice diet without tobacco tar, also similarly supplemented with vegetables and water. 0.6 g of p-dimethylaminoazobenzene (DAB) was dis-

solved in 30 cc of soya bean oil and the solution was evenly mixed with 1 kg of the tar and control diets described above alike, and rats were allowed to feed on the mixture ad libitum. The amount of DAB was 0.06 per cent of rice diet (dry weight) throughout the period of 4 months. After the completion of DAB feeding, both groups of animals were placed on a rice diet without the carcinogen and tobacco tar for additional one month.

Records were kept of the average amount of food consumed and the average body weight during the feeding period. The average amounts of diet per rat per day were 7.0–12.1 g in the tar group and 9.7–10.0 g in the control group respectively.

During the early course of the experiment, the weight and growth rates of animals in the tar group were appreciably lower than those for the controls, but no explanation is available for this pheonomenon at the present time. It could be due either to an inadequate intake of food or to the decreased utilization of food, as is shown in Fig. 1. But the body weight of the tar fed rats was increased rapidly just after the removal of the dye and tobacco tar from the diet. Toward the end of the experiment, little difference in the body weight was revealed between the animals in both groups.

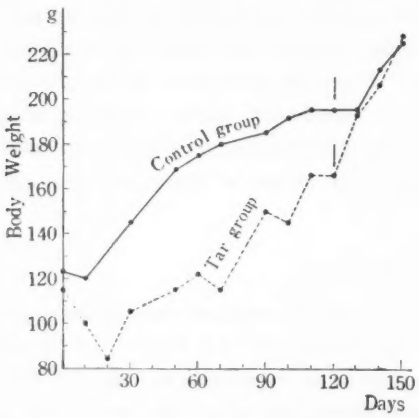

Fig. 1. Curves showing the body weight of rats surviving 150 days (Experiment 2). Arrow shows the time when dye and tobacco tar was discontinued.

Some of the rats died early in the course of the experiment without showing noteworthy changes. Experiment was terminated at 150 days after the beginning, by killing all the rats of the both groups then surviving and by performing autopsy.

The experiment was repeated twice (Experiments 1 and 2), at each time 40 rats having been devided in two groups which were fed on one or the other of the above mixtures.

The results of the above experiments are summerized in Table 1, where the nature of liver findings is tabulated in the following four degrees: liver cancer with marked annular cirrhosis, cirrhotic changes only, slightly uneven surface without extensive proliferation of connective tissue, and macroscopically normal. The identification of these liver findings has been described in detail in the publication cited (11).

In the first group (tobacco tar fed), 20 (71.5%) of the total 28 rats showed apparently normal liver, in the 3 cases (10.7%) the liver changes were not advanced

Table 1. Comparison of Liver Changes between the First Group (tobacco tar fed) and the Second (control) Group.

Experimental group	No. of Experiment	No. of rats surviving 150 days	Liver Findings			
			Macroscopically normal liver	Liver with slightly uneven surface	Cirrhotic liver	Liver cancer
First group	1	18	10	3	4	1
(tobacco tar fed)	2	10	10	0	0	0
Total		28	20(71.5%)	3(10.7%)	4(14.3%)	1 (3.5%)
Second group	1	12	0	1	0	9+2*
(control)	2	12	2	1	1	6+2*
Total		24	2 (8.3%)	2 (8.3%)	1 (4.2%)	19(79.2%)

* Liver cancer with metastases.

beyond the stage of somewhat granular and uneven surface, in the 4 cases (14.3%) the liver were cirrhotic only and in the remaining 1 case (3.5 %) the hepatic change was advanced to warrant the diagnosis of cancer with a small cancerous nodule. Especially in the second experiment, it is norteworthy that in all 10 rats livers remained macroscopically normal even at 150th day.

In the marked contrast to the conditions in the first group, the second group (control) included 19 cases (79.2%) of liver cancer accompanying annular cirrhosis, four of them with metastases in the lung, 1 case (4.2%) of typical cirrhosis, only 2 cases (8.3%) of liver with slightly uneven surface and the remaining 2 cases (8.3%) of normal liver macroscopically.

The above result leaves little doubt as to the marked inhibiting effect which tobacco tar at the level of 2.5% in the diet exerts on the production of liver cancer by oral administration of DAB.

Experiment 3: From the result of the experiments above, it has become of interest to determine whether or not the tobacco tar would inhibit the production of liver cancer when DAB was injected into instead of fed to rats. The following experiment to be reported was undertaken in order to elucidate this point.

Two groups of 15 albino rats of Wistar strain each were used in the experiment. The first group (tobacco tar fed) was kept on the rice diet, containing of 2.5 per cent solution of whole tobacco tar, just same as used in the above experiments. The second group (control) was maintained on rice diet without tobacco tar. All the rats in both groups were given injections of 2.5 per cent solution of DAB in soya bean oil in 0.5 cc amounts (i. e., 12.5 mg of DAB per injection). Injections were made intraperitoneally at the left hypogastric region, and were repeated every week for the total duration of the experiment. More than half of rats died

early in the course of the experiment among the tar fed rats. The experiment was terminated 200 days after the beginning by killing all the rats in order to make a final comparison of liver changes between the two groups. There were only 4 tobacco tar fed and 9 control rats at this time. The liver findings of rats of these two groups are summarized in Table 2.

Table 2. Comparison of Liver Changes between Tar fed and Control Groups, 200 days after the Beginning of the Experiment (Amount of DAB injected: 350 mg).

Experimental groups	No. of rats surviving 200 days	Liver Findings			
		Macroscopically normal liver	Liver with slightly uneven surface	Cirrhotic liver	Liver cancer
Tobacco tar group	4	2 (50.0%)	2 (50.0%)	0	0
Control group	9	3 (33.3%)	2 (22.2%)	1(11.1%)	3(33.3%)

In the tar fed group 2 (50.0%) of 4 rats, 2 rats showed apparently normal liver and in the remaining 2 the livers (50.0%) were slightly granular. In no case these hepatic changes were advanced to warrant the diagnosis of cirrhosis or cancer. The control group included 3 cases (33.3%) of liver cancer, 1 case (11.1%) of typical cirrhosis, 2 cases (22.2%) of uneven surface and 3 cases (33.3%) of macroscopically normal.

The result of the experiment may be regarded as the evidence that tobacco tar inhibits the production of liver cancer by the repeated injections of DAB. It must be added, however, that the degree and extent of the inhibiting effect of tobacco tar was not so complete as when DAB was given orally in the Experiments 1 and 2.

Discussion

Since the discovery of the carcinogenic action of azo-compounds, it was found that the rate of tumor formation was markedly altered by the addition of various materials, protein, vitamins, fat and other factors, to the diet. Much of the early literature on the subject has been reviewed by previous investigators (12–18). But, as far as the authors are aware, no such drastic inhibition as effected by feeding liver and kidney has ever been brought about by any other dietary means.

In the present experiment, it is obvious that the supply of the whole condensate of tobacco to the rice diet brought about a considerable inhibition of liver cancer production by DAB, although the degree of the inhibition was not as high as that attained by liver feeding.

It is not entirely without reason to suspect that the tobacco tar may act simply

as a detoxicant by either combining with or decomposing DAB so as to render it noncarcinogenic. But the nature of the tobacco tar constituents which may be responsible for this marked inhibition of liver cancer production remains undetermined.

Several workers demonstrated that the application of carcinogenic hydrocarbons resulted in a lowering of liver cancer incidence in rats fed diets containing hepatic carcinogen (19–26). Recently it is reported that the repeated injection of trypan-blue reduced the incidence of liver cancer by DAB (27–29). On the other hand, the carcinogenicity of tobacco smoking or cigarette tar has been reported. More recently, Cooper and Lindsey have confirmed the presence of 3 : 4 benzpyrene, 1 : 12 benzpyrene and other hydrocarbons in the tobacco smoke (30). Then, there is a possibility that these hydrocarbons in the tobacco smoke may have an inhibiting action on the carcinogenesis by DAB when applied simultaneously. Moreover, we must assume that such an inhibiting factor might be in the alkaloid fraction of the tars because the whole condensate was used in the experiment. The nature of the substance responsible for the inhibition remains an open question, which can be settled only through further experiments.

Conclusion

The addition of the cigarette tar to the diet brings about a definite inhibition of liver cancer production by p-dimethylaminoaobenzene administered per os or intraperitoneally. How the tobacco tar affects the experimental production of liver cancer is indeed a complex problem, and an investigation on this question is now under way in this laboratory.

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要　旨

タバコタールによる実験的肝癌生成抑制実験

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p-Dimethylaminoazobenzene 飼与あるいは腹腔内注射による肝癌生成実験に際して，巻タバコより得た粗タールを2.5%の割合に飼料に添加すると著しい抑制作用を示した。粗タール中の如何なる物質が抑制的に働くのであるか将来の研究に待ちたい。

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